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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: SOME BASIC STUDIES AND APPLICATION IN MICROBIAL TRANSFORMATION

THESIS

Submitted by
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for the degree of Doctor of Philosophy of
the University of Bath

1987

This research has been carried out in the School of Pharmacy and Pharmacology of the University of Bath under the supervision of T.M.Jefferies, *B.Pharm., M.Pharm., Ph.D., M.P.S.*, C.J.Soper, *B.Pharm., M.Sc., Ph.D., M.P.S.* and Professor R.T.Parfitt, *Ph.D., F.P.S., Ch.Chem., F.R.I.C.*

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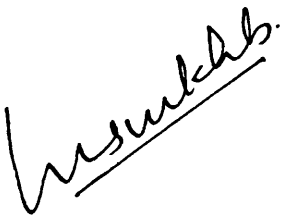

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D E D I C A T I O N

This thesis is dedicated to my late father.

SUMMARY

In Chapter 1 a general introduction to some of the basic problems that are encountered in microbial transformation studies and also those aspects of reversed-phase HPLC which needs to be studied further are outlined.

Chapter 2 describes the application of reversed-phase HPLC analysis to the preliminary identification of microbial transformation products of test benzodiazepines. These identified products are then compared with the metabolites of the same test compounds observed in humans. A significant correlation was observed suggesting that the fungus Cunnunghamella bainieri mimicks human biotransformation of 1,4-benzodiazepines to a large extent.

Several solvent strength parameters available for the quantitation of the eluotropic strength of the mobile phase in RP-HPLC are reviewed in Chapter 3. A new solvent strength parameter base on the hydrophobic property of the solvent has been proposed. The n-octanol/water partition coefficient of a solvent has been suggested as a solvent strength parameter and its use resulted in relatively precise predictions of mobile phase compositions which are iso-eluotropic in reversed-phase HPLC. A comparison has been made between the compositions predicted by this study and other strength parameters including empirical transfer

rules. The unique advantages offered by the use of Partition Coefficient for the calculation of the strength parameter of mixed solvents has been discussed. Analysis of experimental as well as literature data supports the view that n-octanol/water logP of the solvent could be used for eluotropic strength calculations.

The retention behaviour of solutes in reversed-phase chromatography has been studied in Chapter 4. A stochastic model based on experimental data has been developed for those systems where hydrophobic interaction is the dominant retention mechanism. The newly proposed solvent strength parameter (Chapter 3) and the n-octanol/water partition coefficient of solutes were found to be significant factors controlling chromatographic retention in RP-HPLC. The model was able to describe the variation in retention by the solute and solvent physico-chemical parameter to a large extent for different but related chromatographic systems. An unique advantage offered by this approach is that it allows all retention data, for all solutes analysed under different solvent systems, to be combined in a single model. It is suggested that this model could be used in method development for those solutes for which n-octanol/water partition coefficients are known.

Optimisation of the chromatographic selectivity in the limited solvent parameter space is considered in Chapter 5. An optimisation strategy employing a

factorial experimental design, bi-dimensional polynomial interpolation and sequential simplex search has been proposed for the optimisation of selectivity in the solvent space defined by iso-eluotropic solvent vectors, employing composite criterion as the response function. Predicted optima showed close agreement with the experimental data. Application of the proposed methodology for optimisation using overlapping resolution map criterion and chromatogram simulation are discussed.

CONTENTS

CHAPTER 1

INTRODUCTION

Chromatographic methods of analysis are well-known for their ability to analyse sample mixtures efficiently as compared to other conventional techniques. Among the chromatographic techniques, however, High Performance Liquid Chromatography (HPLC) has aquired an unique place due to its rapidity of analysis, low sample consumption, high degree of selectivity and milder conditions of analysis so that thermolabile substances can be analysed. Additionally the availability of the high performance chemically modified monolayer bonded phases with small particle diameter has made Reversed-Phase HPLC one of the most widely used analytical tools[1.1]*. The advantages that RP-HPLC has over Normal-Phase HPLC are its stability, availability of a variety of bonded stationary phases and relatively low organic solvent requirement.

In liquid chromatographic systems the retention of a solute is related to its physico-chemical nature and therefore its thermodynamic properties in the solution.

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* Reference numbers are given in square brackets.

In other words, some of the thermodynamic or physico-chemical properties of solutes can be established from their chromatographic retention[1.2]. This relationship has been exploited further to correlate chromatographic retention to biological activity and bioaccumulation[1.3]. Therefore HPLC does not only offer an efficient analytical tool but also provides a possibility of obtaining physico-chemical parameters of the analytes.

At Bath University, as a part of a long-term project work has been carried out to explore and to exploit the potential of microorganisms either for chemical conversion for commercial exploitation or to obtain microbial metabolic models for the transformation of drugs and in the study of drug toxicity. Sewell[1.4] carried out screening of various species from genus Cunninghamella and Pseudomonas against different model drug compounds. Characteristically, in such screening experiments a large number of chemicals/drugs are tested against an even larger population of microorganisms. Furthermore, identification and quantitation of transformation products would be necessary so that such information can be used to select the microorganism(s) providing the most favourable transformation. Some of the practical problems that are encountered in such studies are:

1. The development of analytical methods suitable for the determination of drugs and their metabolites in biological fluids.

2. Qualitative and quantitative differences in biotransformation routes exist between humans and other mammals.

3. The procurement of sufficient quantities of metabolites for structural elucidation and biological testing. More importantly for those metabolites which are difficult to synthesise.

4. The availability of appropriate animal models, animals or volunteers for specific metabolic studies.

In a general screening programme there may be a large number of microbial species tested against a variety of substrates. Therefore, in such situations establishing the identity of each metabolite as obtained from each specie for every substrate is an enormous task. However, it may not be necessary to establish the full identity of all components of the transformation mixture unless there is some evidence of the presence of the product(s) of interest. In other words, a preliminary analysis may be sufficient to select those species which provide significant transformation(s). It is probable that a product/specie may go undetected due to the nature of the preliminary investigation. Neverthless, this is the risk which may be balanced against the time and cost factor involved for the entire screening programme.

It was therefore of interest to investigate if reversed-phase high performance liquid chromatography (RP-HPLC) could be employed for the needs stated above. RP-HPLC has been employed[1.2] for the determination of physico-chemical parameters from the retention of unknown compounds and has the advantage that quantitation may be done relatively quickly. Therefore using this analytical technique some physico-chemical parameter of the unknown transformation product may be obtained.

Previous work at Bath[1.5] was conducted on the microbial N-dealkylation of some 1,4-benzodizepines by Cunninghamella sp. In the present work further studies were undertaken aimed at comparing the biotransformation of diazepam, medazepam and flurazepam by Cunninghamella bainieri with that observed in humans using an RP-HPLC method of analysis for obtaining the preliminary identification of the transformation product(s).

Some aspects of reversed-phase HPLC which became obvious for further studies were (1) to investigate eluotropic strength of the solvents so that a reliable estimate of solvent strength for RP-HPLC could be obtained and (2) to examine chromatographic behaviour of solutes in relation to their hydrophobic parameter so that a general hydrophobic interaction model can be delveloped. It was therefore, thought that a systematic study of these and other aspects, such as solvent optimisation, would be valuable.

Various workers have proposed different solvent "strength" parameters such as P'[1.6], S[1.7], δ(T)[1.8] and also empirical transfer rules[1.9] for reversed-phase LC. Research workers have made use of the parameters proposed for reversed-phase LC, although these parameters have some limitations and do not provide adequate flexibility in their routine use as desired by chromatographers.

It was therefore thought of interest to investigate if a parameter could be found that would overcome the difficulties faced by chromatographers in solvent transfer calculation to obtain iso-eluotropic compositions.

Prediction of the conditions required in reversed-phase high performance liquid chromatography (RP-HPLC), for the adequate resolution of a particular group of analytes could be most useful, however it is equally elusive. Ideally, it should be possible to calculate the retention parameter of a given analyte in the chosen column - mobile phase system from the physico-chemical properties of the analyte, mobile phase and column.

Recently, the selection of the initial mobile phase composition has been improved by the introduction of systematic, rather than "trial-and-error", processes. These approaches include the use of a gradient run in order to select an isocratic composition[1.10], an iterative method[1.11], and an automated systematic "trial-and-error" type algorithm (simplex)[1.12,1.13].

These methods do not require a priori knowledge of the physico-chemical properties of the solutes. Other workers have approached the problem by considering the physico-chemical properties of the analytes and using semi-deterministic or semi-empirical techniques.

In the present work partition coefficients of analytes and mobile phases have been used to calculate the retention parameter of any un-ionized analyte in any mobile phase system.

The factors that influence the analysis are the nature of the stationary phase, the qualitative and quantitative composition of the mobile phase, the mode of separation i.e. isocratic or gradient elution, flow rate, temperature, pH, buffer concentration and in the case of ion-pair chromatography, the concentration of ion-pair reagent. Considering the many variables concerned it is not surprising that the development of an HPLC method is a challenge to chromatographers.

Normally many of the above mentioned factors are preselected and maintained at a constant level whilst changing only those factors which potentially offers maximum variation in the selectivity. Generally, changes in the retention and selectivity (α) are offered by altering the qualitative and quantitative composition of the mobile phase. Solvent strength/selectivity changes offers a number of advantages in resolution of the solutes. Therefore once other parameters are selected, from previous experience or other systematic approaches, the aim of

the analyst is to obtain optimum analysis by altering the qualitative and quantitative composition of the mobile phase, i.e. solvent optimization. Therefore the problem of finding the appropriate composition of the solvent, within the solvent parameter space is at the heart of chromatographic selectivity optimisation.

Traditionally the optimisation of binary solvent systems has been achieved by "trial-and-error" methods. This is probably an efficient way of method development if the sample mixture consists of a few chemically distinct compounds, using binary solvents. However the difficulty of the analysis increases steeply for complex mixtures and where either ternary or quaternary solvent systems are required or employed. In such cases the traditional approach either cannot be used or is too inefficient to use practically.

One of the earliest attempts was to use a graphical technique[1.14], known as "window diagram" for the optimisation of stationary phases in GLC, which is in some way similar to the solvent optimisation in RPLC. However, with the advent of computers, especially the microcomputer and microprocessor controlled liquid chromatographs, which allowed automated but controlled analysis, made it possible to use some of the established mathematical, statistical or numerical techniques to attack the optimisation problem in HPLC.

Although there are a number of optimisation methods which are available[1.15] they have their distinct advantages as well as limitations. It was therefore decided, in the present work, to explore different possibilities.

CHAPTER 2

APPLICATION OF REVERSED-PHASE HPLC IN THE STUDY OF MICROBIAL TRANSFORMATION

2.1 INTRODUCTION

The metabolism, or more strictly the biotransformation of drugs and other chemicals in mammals has been extensively studied. These studies generate information on the mechanism of action and/or toxicity of chemicals in general and drugs in particular. Studies regarding the mammalian and especially human biotransformation of drugs is vital in the pharmaceutical field. Some of the practical problems that are encountered in such studies are:

1. The development of analytical methods suitable for the determination of drugs and their metabolites in biological fluids.

2. Qualitative and quantitative differences in biotransformation routes exist between humans and other mammals.

3. The procurement of sufficient quantities of metabolites for structural elucidation and biological testing. More importantly for those

metabolites which are difficult to synthesise.

4. The availability of appropriate animal models, animals or volunteers for specific metabolic studies.

It has been suggested[2.1], that it might be possible to define microbial transformation systems that could mimic many biotransformations observed in mammals/humans. Such transformation systems could be either a group of microrganisms or a single microrganism. When employed, such systems could provide sufficient quantities of the metabolites of interest for further studies, by well-established fermentation techniques. There are a number of potential benefits of employing microbial methods to synthesise or derivatise drugs, as opposed to chemical methods. Primarily, microbial transformations may give rise to increased and more consistent yields. They are usually specific with respect to a particular substrate or a reaction type[2.2], and some reactions that are chemically not possible may be mediated by microorganism[2.3]. It may even be possible to achieve several coupled reactions in one transformation step[2.3]. Additionally the transformations are conducted under milder conditions and in aqeous media with cheaper raw materials when compared to chemical synthesis.

Microbial transformation may also be exploited commercially in the manufacture of pharmaceuticals where chemical synthesis requires the use of toxic or hazardous reagents. Chemical modification of steroids[2.4-2.7], alkaloids[2.5,2.8] and antibiotics[2.9,2.10], has been successfully achieved using microbial transformation.

Previous workers at Bath University[2.11,2.12] envisaged that a microbial transformation system, capable of effecting the N-dealkylation could be developed to overcome the difficulties faced by medicinal chemists in preparing useful drug intermediates, and could also be valuable in drug metabolism studies. Gibson[2.12] studied N-dealkylation of 1,4-benzodiazepines using Cunninghamella sp. following the findings of Sewell[2.10] that several species of genus Cunnighamella were able to N-dealkylate most of the chosen substrates with N-alkyl functionality. This conclusion was reached after extensive screening experiments on model compounds such as amytriptiline, chlorpromazine, des-methyl chlorpromazine, codeine and diazepam, employing various species of Streptomyces and Cunninghamella

It is evident from the preceding discussion that the choice of microorganism(s) for either a metabolic model or for a specific chemical conversion is crucial. Therefore it is essential to carry out extensive screening tests on either model compounds or specific

chemical entities so that microbes may be identified that provides efficient, in terms of quantity and specificity, transformation. This process of selection requires (1) fermentation experiments involving various substrates and different species of microorganisms, and (2) analysis of the fermentation products such that the identities of the transformation products are obtained. From the information that could be gained from these experiments a judgement may be made regarding the specificity and efficiency for conversion of a substrate or in case of checking suitability as a microbial model, a comparison can be made with available data on the mammalian metabolites.

In a general screening programme there may be a large number of microbial species tested against a variety of substrates. Therefore, in such situations establishing the identity of each metabolite as obtained from each specie for every substrate is an enormous task. However, it may not be necessary to establish the full identity of all components of the transformation mixture unless there is some evidence of the presence of the product(s) of interest. In other words, a preliminary analysis may be sufficient to accept those species which provide significant transformation(s). It is possible/probable that a product/specie may go undetected due to the nature of the preliminary investigation. Neverthless, this is the risk which may be balanced against the time and cost factor involved for the entire screening programme.

It was therefore of interest to investigate if reversed-phase high performance liquid chromatography (RP-HPLC) could be employed for the needs stated above. RP-HPLC has been employed[2.13] for the determination of physico-chemical parameters from the retention of unknown compounds and has the advantage that quantitation may be done relatively quickly. Therefore using this analytical technique some physico-chemical parameter of the unknown transformation product may be obtained.

Previous work at Bath[2.12] was conducted on the microbial N-dealkylation of some 1,4-benzodizepines by Cunninghamella sp. In the present work further studies were undertaken aimed at comparing the biotransformation of diazepam, medazepam and flurazepam by Cunninghamella bainieri with that observed in humans using an RP-HPLC method of analysis for obtaining the preliminary identification of the transformation product(s).

2.2 EXPERIMENTAL

2.2.1 Fermentation Studies

The growth and transformability of submerged cultures of Cunninghamella sp. have been demonstrated in previous studies by Sewell[2.11] on Cunninghamella echinulata and Gibson[2.12] on Cunninghamella bainieri. Codeine and 1,4-benzodiazepines were N-dealkylated with high reproducibility when a two-stage growth and incubation protocol was employed. Therefore, in the

present work this protocol was employed.

2.2.1.1 Materials

Microorganism

Cunninghamella bainieri: C43, obtained from The American Cyanamid Company, Lederle Laboratories, New York, USA.

The microorganism stored under liquid nitrogen was transfered to Malt Extract Agar plates and incubated for 10 days at 27°C. The resultant spores were harvested in saline and stored at 4°C.

Growth Media

1) Stock Plates

Malt Extract Agar (Oxoid CM 5a) was prepared according to the manufacturer's directions and sterilised at 121°C for 15min. The medium was then poured into petri dishes and stored at 4°C.

2) Chemically Defined Growth Media

These were prepared from Analar grade reagents(BDH ltd.) and glass distilled water.

Stage-one Basal Medium : Salts(mg/l);

KH_2PO_4(378.0), $Na_2HPO_4.12H_2O$(989.0), $(NH_4)_2SO_4$(667.0), $MgSO_4.7H_2O$(28.0), $CaCl_2.2H_2O$(5.6), $ZnSO_4.7H_2O$(22.0), $MnSO_4.7H_2O$(2.20), $CuSO_4.5H_2O$(0.56), $FeSO_4.7H_2O$(11.0), Na_2SO4(56.0), $NaMoO_4.2H_2O$(5.60), disodium EDTA(66.70).

The above medium was prepared as a double strength solution, packed in 500ml bottles and autoclaved at 121°C for 20min.

Stage-two Basal Medium :

Salts (mg/ml); K_2HPO_4(3327.0), $NaH_2PO_4.2H_2O$(732.0), $(NH_4)_2SO_4$(500.0), $MgSO_4.7H_2O$(290.0), disodium EDTA(290.0).

Trace elements (mg/ml); $CaCl_2.2H_2O$(33.0), $(NH_4)_2MoO_7.4H_2O$(93.0), $FeSO_4.7H_2O$(3.50), $ZnSO_4.7H_2O$(5.50), $MnSO_4.4H_2O$(0.75), $CuSO_4.5H_2O$(0.20), $Co(NO_3)_2.6H_2O$(0.125), $Na_2B_4O_7.10H$ O(0.09).

The salts mixture was prepared as a double strength solution and was sterilised by autoclaving at 121°C for 20min. The trace elements were dissolved in glass distilled water to produce a 50X concentrate and the solution was sterilised by filtration using 0.2μm pore membrane.

Caseine Hydrolysate Solution

A 10%w/v solution was prepared by dissolving caseine

hydrolysate poweder (Oxoid L4C) in hot distilled water. It was sterilised by filtration and stored at 4°C.

Glucose solution

Glucose(BDH) was dissolved in distilled water to make a 10%w/v solution and was sterilised by filtration.

Transformation Substrates

Diazepam, Medazepam and Flurazepam were gifts from Roche Products Ltd.

2.2.1.2 Methods

1) Inoculum Preparation

In a 250ml flask containing 50ml of sterile saline one half of the incubated stock agar plate was suspended and few sterile glass beads were added. The mixture was stirred vigorously on a vortex mixer for 5min and allowed to settle. The supernatant, containing spores, was transfered to sterile centrifuge tubes and centrifuged at 2000rpm for 2min. The spore count of the supernatant was adjusted to about 30 000 000/ml.

2) Stage-one Culture

Stage-one culture medium consisted of Stage-One Basal Medium(25ml), Caseine Hydrolysate Solution(5ml), Glucose Solution(5ml) and sterile distilled water (to

50ml). This medium, contained in 250ml siliconised, fluted flask, was inoculated with 2ml of spore inoculum. Cultures were then incubated at 27°C with rotary agitation at 250rpm for 48hr. The contents of the flasks were pooled and diluted to obtain about 6 mg/ml dry cell weight.

3) Stage-two Culture

5ml aliquots of the Stage-one Culture were transfered with Kipps burette(5ml) to fluted, siliconised flasks, containing Stage-two Basal Medium (25ml), Caseine Hydrolysate Solution(5ml), Glucose Solution (5ml) and sterile distilled water (to 50ml). Substrate solution was added to the flask to give a final concentration of 1mM. Cultures were incubated at 27°C with rotary agitation at 250rpm for seven days. A schematic diagram for the fermentation protocol is shown in Fig.2.1.

2.2.2 HPLC Analysis

Chromatographic analyses were performed using a modular system assembled from a Constametric III pump (Milton Roy, Stone, UK), Rheodyne 7125 injection valve and a UV-LC detector (Pye-Unicam). A 150x4.6mm i.d. column packed with 6μm ZORBAX-ODS (DuPont) was employed as reversed-phase stationary phase and analyses were carried out at 35°C with mobile phase flow rate of 2ml/min. Effluents were monitored at 254nm.

The mobile-phase consisted of methanol, tetrahydrofuran (Fisons, UK) and 0.05M phosphate buffer (pH 6.5). The proportions of the organic modifiers and buffer (MeOH;THF;Buffer, %v/v) employed for the analysis of transformation products for each substrate were: Diazepam(30;15;55, %v/v), Medazepam(30;20;50, %v/v) and Flurazepam(34;17;49, %v/v). The substrates are abbreviated as DZP, MZP and FZP for convenience. Using these solvent systems analyses were also carried out for clonazepam, DZP, MZP and FZP for obtaining relationships between logk' and logP.

2.3 RESULTS AND DISCUSSION

The data in Table 2.1 shows that all the substrates, viz. DZP, MZP and FZP, were tranformed by Cunninghamella bainieri to give products. These products were not detected in the control flasks. Therefore, it indicates that the products observed in the test flasks were due to transformation rather than degradation due to other factors such as pH, temperature etc.

In order to establish their identity it was necessary to obtain a parameter which could be easily obtained so that a comparison may be made with similar parameter of the known metabolites. It is known that in RP-HPLC the retention of a solute is mainly governed by its hydrophobic property[2.14]. On this basis several research workers have reported[2.15] either a correlation of hydrophobic parameter, n-octanol/water

logarithm of partition coefficient (logP), or predictions have been made for known solutes from their logP parameter values. Since in the present work the analytical conditions were chosen to provide mostly hydrophobic dominated retentions it was possible to apply accepted relationship between retention (logk') and partition coefficient (logP). But before the hydrophobic parameter of each transformation products can be estimated it was necessary to create a calibration of logP vs. logk'. For this reason analyses were performed using known compounds, viz. clonazepam(2.477), DZP(2.993), MZP(3.970) and FZP(4.430), whose logP values were known(values given in parentheses). In the present work the theoretical logP values were calculated according to Rekker's fragmental approach[2.16]. A typical calculation is shown in Fig.2.2. It is apparent that the theoretical value shows close agreement with the experimentally determined value. The reason for employing theoretical rather than experimental values is as follows. Since the experimental logP values are available for only few benzodiazepines it would not be possible to find the logP values of all the metabolites from the literature, or to determine them experimentally, as obtaining these metabolites in sufficient quantity would not be practicable. Therefore in order to maintain consistency all logP values either for known standard or for the known metabolites were calculated.

For each mobile-phase system employed for the different

substrate analysis calibration equations (Eq.2.1) were developed by analysis of clonazepam, DZP, MZP and FZP. The solvent compositions and the correlation coefficient for the following relationship are shown in Table 2.2.

$$\log k' = A + B \cdot \log P \qquad \ldots(Eq.2.1)$$

Where, A and B are coefficients.

It is evident that for all the solvent-systems the correlation is significantly highe so that transfer equations may be employed to predict the logP of a transformation product from its retention. The logP values calculated according to Eq.2.1 are designated by logPk(1) and appear in Table 2.1. It was however felt that this approach may not be suitable for general screening because different solvent systems may have been employed for the given stationary phase, where relationship like Eq.2.1 would require to be established for each solvent system separately.

It is known that the solvent can be described in terms of its eluotropic strength by parameter P′ as originally suggested by Snyder[2.17]. It was therefore decided to incorporate such a parameter in the expression Eq.2.1 and develop a general expression. Therefore parameter P′ was calculated for the mobile phases used and appear in Table 2.2. Retention is known to be function of solvent strength and the hydrophobic parameter of the solutes for RPLC. Considering logk′ as dependent and logP and P′ as independent parameters a general linear expression

(Eq.2.2) can be obtained for the retentions(k′) in the range of 1 to 20.

$$\log k' = A + B \cdot \log P + C \cdot P' \qquad \ldots(Eq.2.2)$$

Where, A,B and C are coefficients.

Where,A=-5.32, B=0.595 and C=0.544 are regression coefficients as obtained from analysis of data in Table 2.1.

This relationship was found to be statistically significant (n=20, r=0.973, s=0.099, F=143) and was able to describe 94.6% of variation in logk′ variable by logP and P′ parameters. A plot of the experimental and calculated logk′ according to Eq.2.2 appear in Fig.2.3. This expression was subsequently used for predicting logP values, designated by logPk(2), of transformation products.The computed logPk(2) values appear in Table 2.1. A comparison of logPk(1) and logPk(2) reveals that they agree to a significant extent. However, it is not yet possible to determine which method of logPk prediction is relatively better as the logP values for the metabolites observed in human were not available for comparison which was one of the aims of this work.

Bridges and Chasseud[2.18] have reported the metabolites and the possible route(s) of their formation for DZP, MZP and FZP in humans. Schematic representations of the metabolites and the routes for their formation for three substrates appear in Fig.2.4,2.5 and 2.6 respectively. Where as the codes

for substrates and their metabolites with the chemical structures are shown in Fig.2.7. From these data, i.e. the structural formula of the possible metabolites, the logP values were calculated by the Rekker approach for each product. The theoretical logP values appear in Table 2.1. Fig.2.2 is a schematic explanation of the calculation of logP.

Having obtained the logP parameter of the metabolites in human it is possible to compare these values with those calculated by Eq.2.1 as well as Eq.2.2, viz. logPk(1) and logPk(2). All these data are presented in Table 2.1 and a graphical comparison is shown Fig.2.8 for logP vs. logPk(1) and in Fig.2.9 for logP vs. logPk(2). Examination of the table and figures suggests that a general agreement between theoretical logP and logPk calculated by the two approaches is obtained. Following is their statistical comparison.

1. logk' vs. logPk(1) : n=17, r=0.980, s=0.131

2. logk' vs. logPk(2) : n=17, r=0.980, s=0.121

3. logPk(1) vs. logPk(2): n=17, r=0.996, s=0.049

It is apparent that the general expression (Eq.2.2) was able to predict the logP values of the metabolites with the same degree of accuracy but with lower variance. when compared to Eq.2.1. This finding suggests that one could employ a general expression like Eq.2.2 for calculation of logP with same degree of accuracy as obtained from calibrations for individual solvent

systems (Eq.2.1).

A significant outcome of this analysis is the high degree of correlation that is observed between the spectrum of human metabolites of benzodiazepines with the transformation products obtained by Cunninghamella bainieri as a model system. This observation suggests that the human biotransformation of the test benzodiazepines is mimicked to a large extent by that of Cunninghamella bainieri. It is also evident that the major transformation product (indicated by '+++' in Table 2.1) is a secondary amine formed probably by a mono-oxygenase system via oxidative N-dealkylation[2.12].

Although this analysis is not a proof in itself as there are other factors which may influence the predictions, neverthless, it does provide a preliminary identification method. Therefore it is believed that this approach could be of value in general screening programmes.

Some aspects of reversed-phase HPLC which became obvious for further studies were (1) to investigate eluotropic strength of the solvents so that a reliable estimate of solvent strength for RP-HPLC could be obtained and (2) to examine chromatographic behaviour of solutes in relation to their hydrophobic parameter so that a general hydrophobic interaction model can be delveloped. It was therefore, thought that a systematic study of these and other aspects, such as

solvent optimisation, would be valuable. In the following chapters some basic aspects of RP-HPLC are considered.

2.4 CONCLUSIONS

The interests of this study were two fold. Firstly, to investigate if RP-HPLC method of analysis could be employed for establishing preliminary identification of the microbial transformation products of 1,4-benzodiazepines and secondly, to compare these identified products with known metabolites of same drugs observed in humans. On the basis of the investigation carried out and the results obtained, following conclusions may be drawn.

1. Cunninghamella bainieri fungus transformed diazepam, medazepam and flurazepam to various products when a two-stage fermentation protocol was used.

2. The preliminary identification showed that each substrate was metabolised mainly to an N-dealkylated product.

3. Employing RP-HPLC it was possible to estimate the hydrophobic parameter, logP, of each substrate and its transformation products.

4. A comparison, based on the hydrophobic parameter, between biotransformation products of test 1,4-benzodiazepines as observed in C. bainieri fungus with those observed in humans showed a significant correlation.

TABLE 2.1

RETENTION DATA (k') OF TRANSFORMATION PRODUCTS, THEIR CALCULATED logPk VALUES AND logP VALUES OF CORRESPONDING METABOLITES OBSERVED IN HUMANS

Compound	k'	(S.D.)	logPk(1)	logPk(2)	logP
		SUBSTRATE DIAZEPAM			
D3	1.81	(0.03)	2.102	2.168	1.828
D2	4.23	(0.08)	2.655	2.787	2.068
D1+++	5.91	(0.11)	2.874	3.031	2.753
DZP	7.09	(0.13)	3.051	3.164	2.993
		SUBSTRATE MEDAZEPAM			
M7	1.85	(0.03)	2.271	2.472	1.823
M6	2.36	(0.03)	2.474	2.650	2.068
M5	3.28	(0.03)	2.753	2.890	2.753
M4	5.07	(0.06)	3.119	3.208	2.993
M3+++	8.61	(0.08)	3.567	3.595	3.316
M2	9.61	(0.23)	3.659	3.674	-----
M1	11.81	(0.20)	3.787	3.825	-----
MZP	13.88	(0.15)	3.932	3.943	3.970
		SUBSTRATE FLURAZEPAM			
F6	2.83	(0.04)	2.641	2.800	2.603
F5	3.58	(0.06)	2.840	2.971	2.642
F4	5.25	(0.08)	3.161	3.250	3.203
F3	6.73	(0.08)	3.371	3.432	3.272
F2	10.09	(0.28)	3.712	3.727	3.528
F1+++	16.54	(0.30)	4.128	4.087	4.107
FZP	23.67	(0.51)	4.230	4.349	4.430

Notes:

1. k'= average of five values.
2. D,M,F with number are codes for the metabolites of DZP, MZP and FZP.
3. logPk(1)=logP calculated from retention using Eq.2.1.
4. logPk(2)=logP calculated from retention using Eq.2.2.
5. ----- Indicates unknown product.
6. +++ Indicates main metabolite.
7. S.D. Standard deviation.

TABLE 2.2

SOLVENT COMPOSITIONS EMPLOYED FOR ANALYSIS OF STANDARDS, THEIR POLARITY AND THE CORRELATION COEFFICIENT FOR Eq.2.1

MOBILE PHASE			POLARITY*	CORRELATION
MeOH%	THF%	BUFFER%	P'	r
30	20	50	7.430	0.997
34	17	49	7.412	0.999
35	20	45	7.175	0.999
40	20	40	6.924	0.999
30	15	55	7.725	0.982

*, $P' = \sum P'(i) \cdot \emptyset(i)$,
P'(i)= Polarity index of ith solvent in mobile phase.
$\emptyset(i)$ = Volume fraction of ith solvent in mixture.

FERMENTATION PROTOCOL

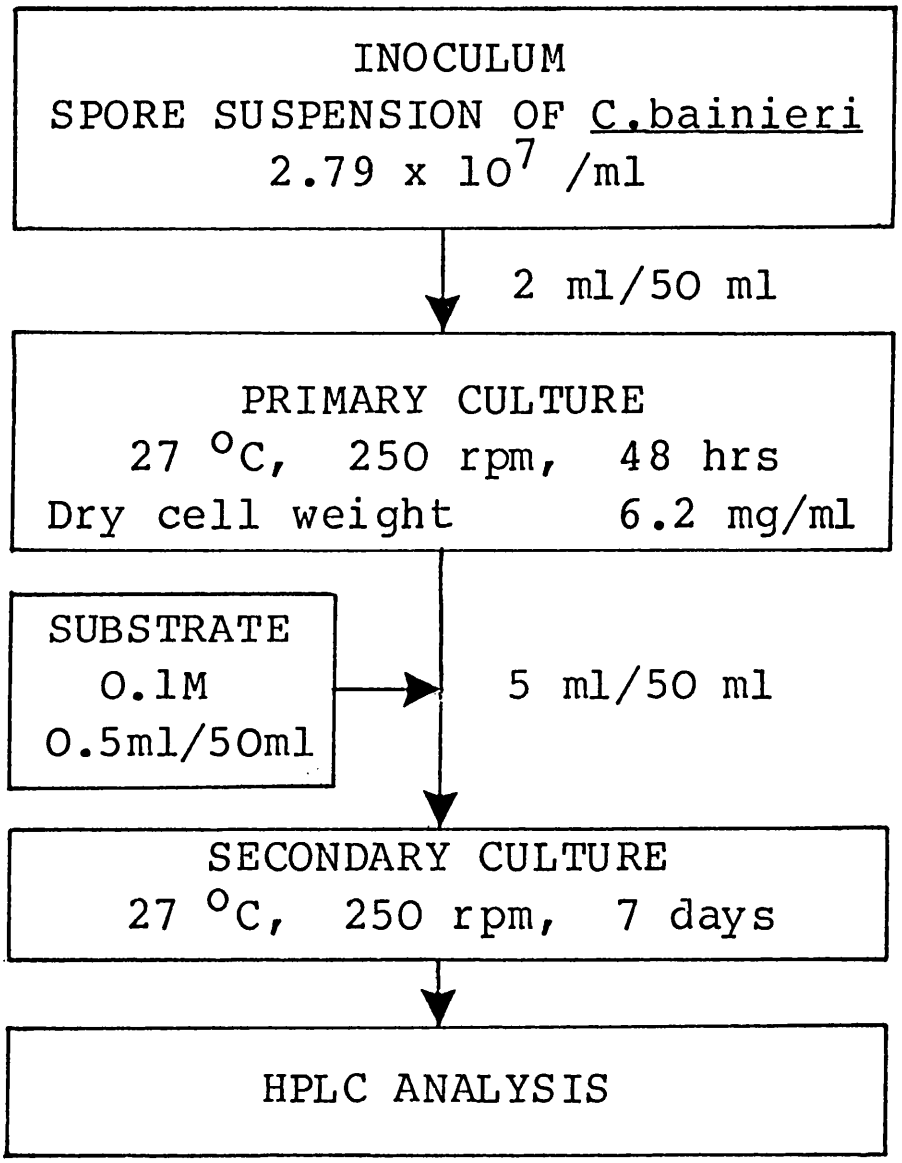
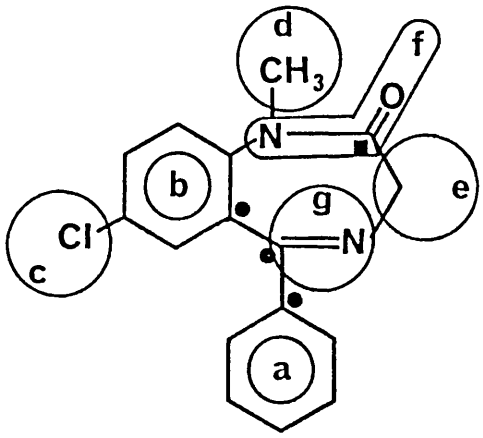


Fig.2.1

USE OF HYDROPHOBIC FRAGMENTAL CONSTANTS TO CALCULATE Log P

DIAZEPAM



	FRAGMENT	FRAGMENTAL CONSTANT(f)
a	C_6H_5	1.886
b	C_6H_3	1.431
c	Ar-Cl	0.922
d	CH_3	0.702
e	CH_2	0.530
f	Ar-NC=O	-1.746
g	Ar-C=N	-1.880
	Cross conjugation (●)	0.287
	Proximity effect(■)	0.861
	Log P =	2.993
	Experimental Log P =	2.802

Fig.2.2

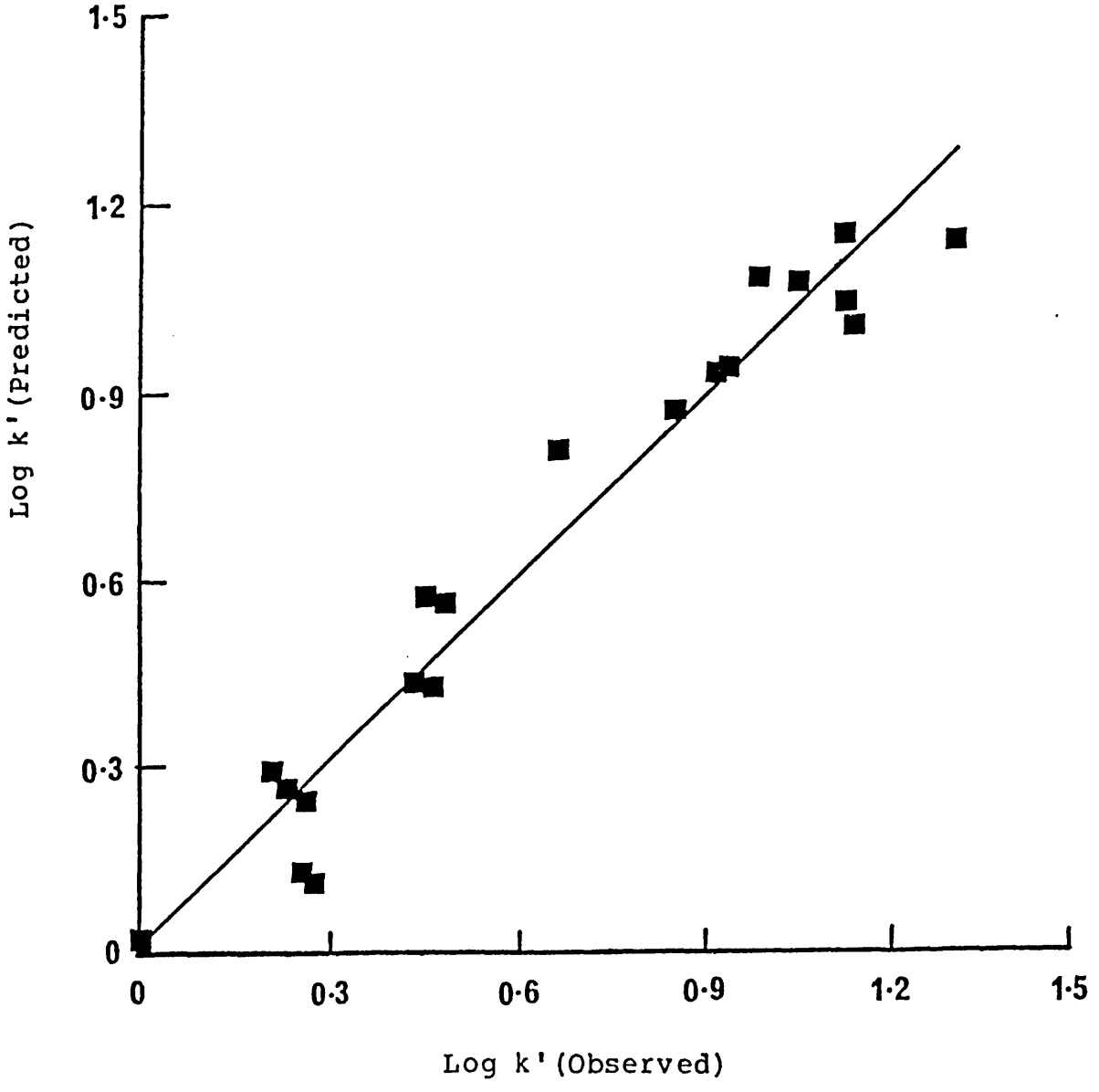


Fig.2.3 Plot of experimental (observed) and calculated logk' according to Eq.2.2.

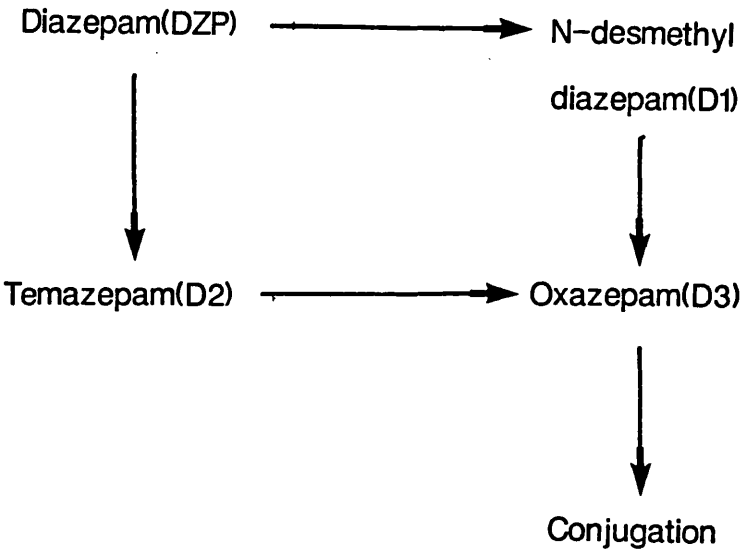


Fig.2.4 Biotransformation of DIAZEPAM in humans[2.18]. Compound code for each metabolite is shown in parentheses.

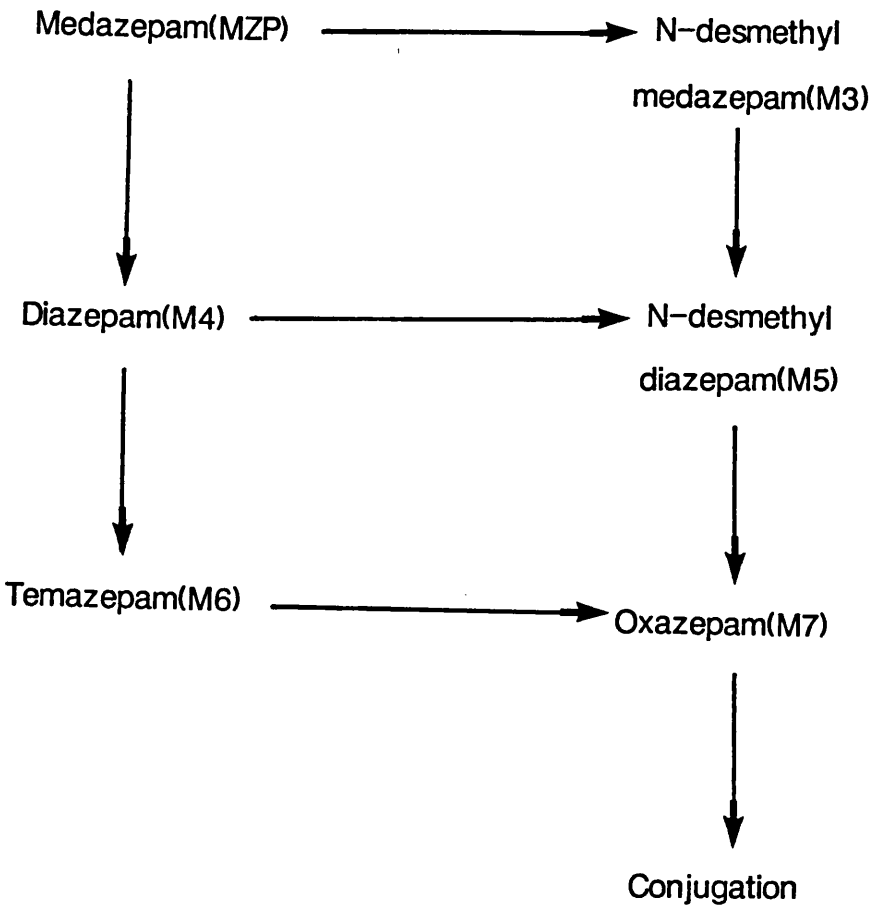


Fig.2.5 Biotransformation of MEDAZEPAM in humans[2.18]. Compound code for each metabolite is shown in parentheses.

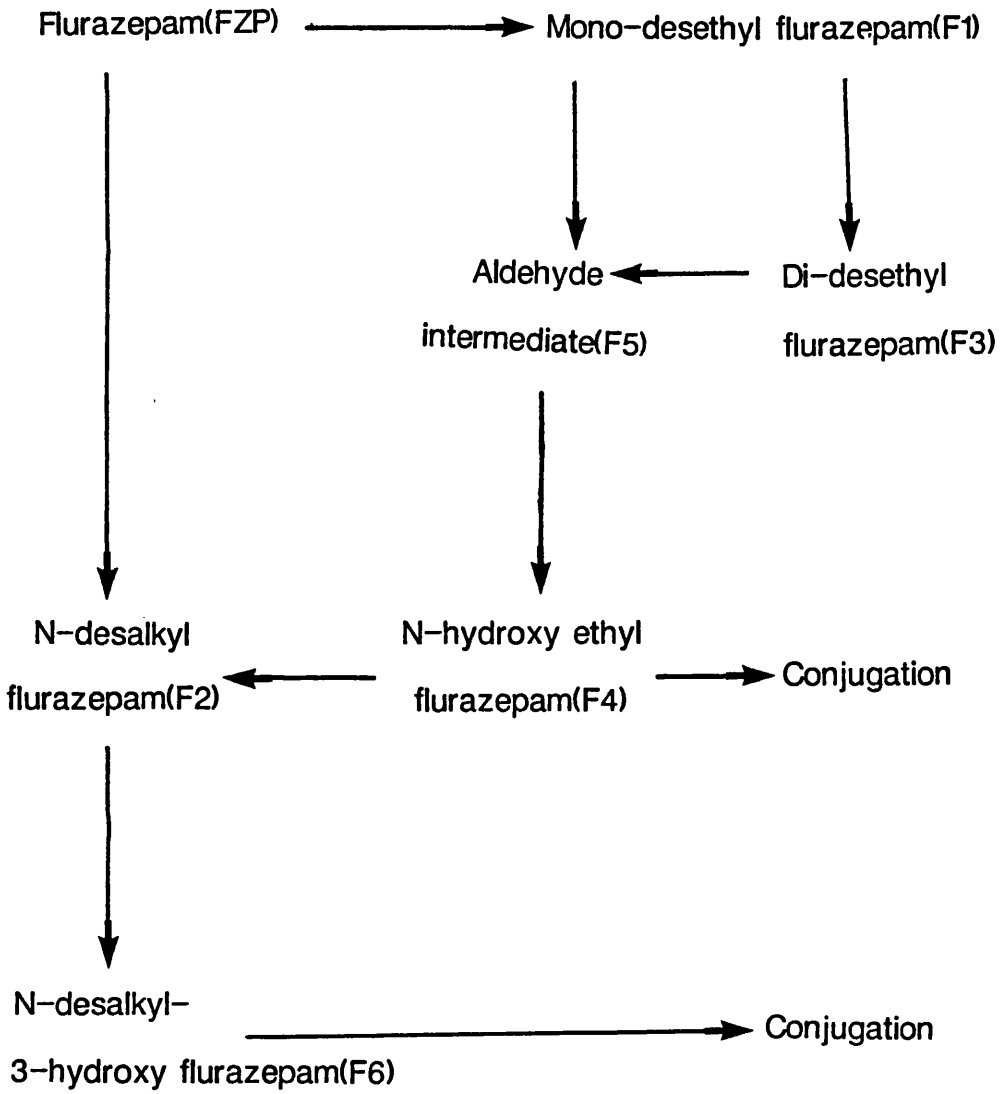
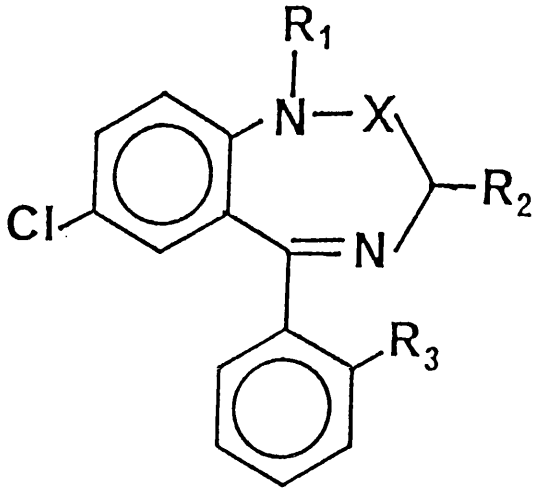


Fig.2.6 Biotransformation of FLURAZEPAM in humans[2.18].
Compound code for each metabolite is shown in parentheses.



Commpound Code	R	R	R	X
D3	H	OH	H	C=O
D2	CH_3	OH	H	C=O
D1+++	H	H	H	C=O
DZP	CH_3	H	H	C=O
M7	H	OH	H	C=O
M6	CH	OH	H	C=O
M5	H	H	H	C=O
M4	CH_3	H	H	C=O
M3+++	H	H	H	CH_2
M2 Unknown	-	-	-	-
M1 Unknown	-	-	-	-
MZP	CH	H	H	CH_2
F6	CH_2CHO	H	F	C=O
F5	H	OH	F	C=O
F4	CH_2CH_2OH	H	F	C=O
F3	$CH_2CH_2NH_2$	H	F	C=O
F2	H	H	F	C=O
F1+++	$(CH_2)_2NHEt$	H	F	C=O
FZP	$(CH_2)_2NEt_2$	H	F	C=O
Clonazepam	H	H	Cl	NO_2

Notes:
1. D,M,F with number are codes for the metabolites of DZP, MZP and FZP.
2. +++ Indicates main metabolite.

Fig.2.7 Structural formulae of substrates and their metabolites.

PLOT OF PARTITION COEFFICIENT (logPk(1)) OF TRANSFORMATION PRODUCTS AND logP OF THE METABOLITES OBSERVED IN HUMAN

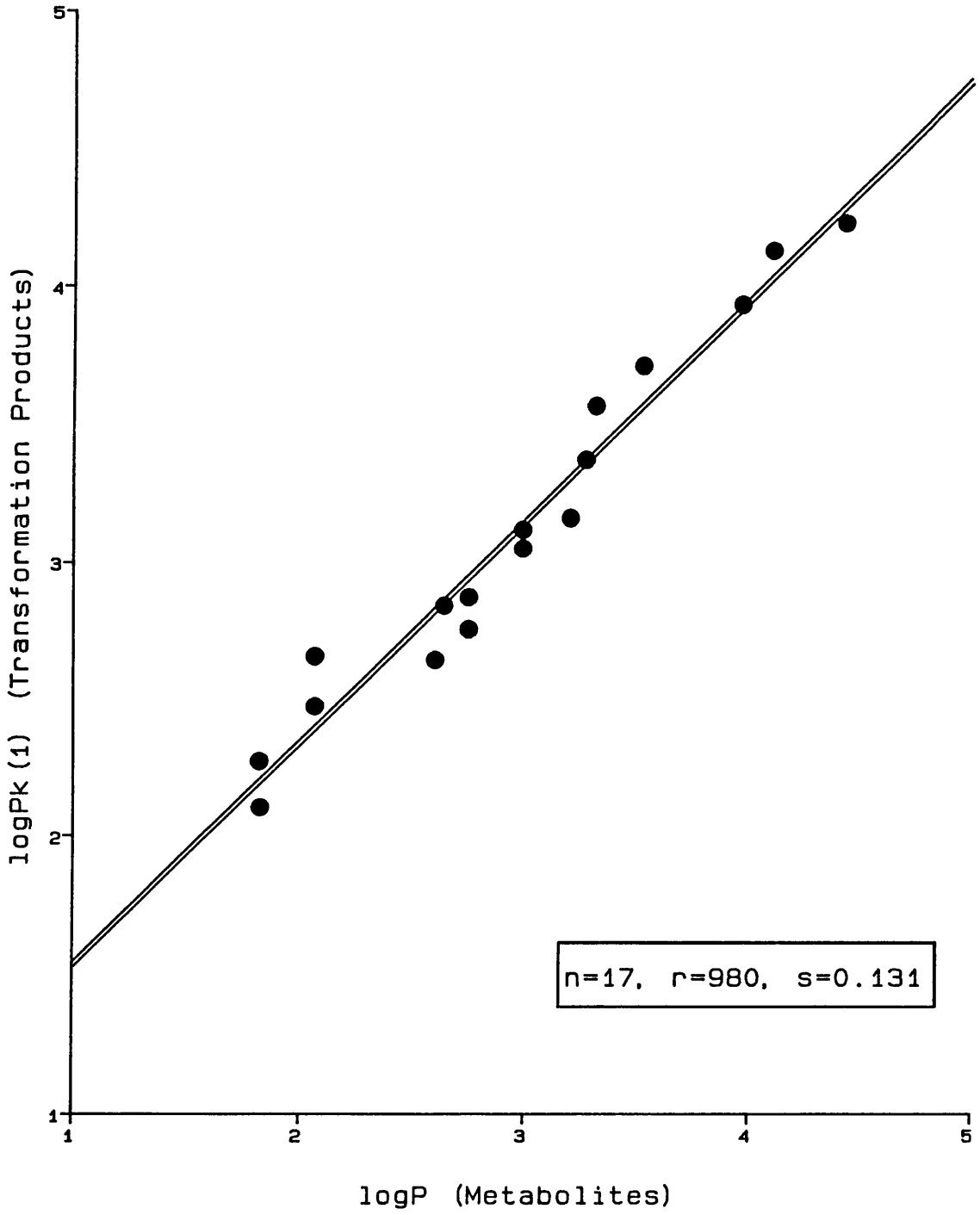


Fig.2.8

PLOT OF PARTITION COEFFICIENT (logPk(2)) OF TRANSFORMATION PRODUCTS AND logP OF THE METABOLITES OBSERVED IN HUMAN

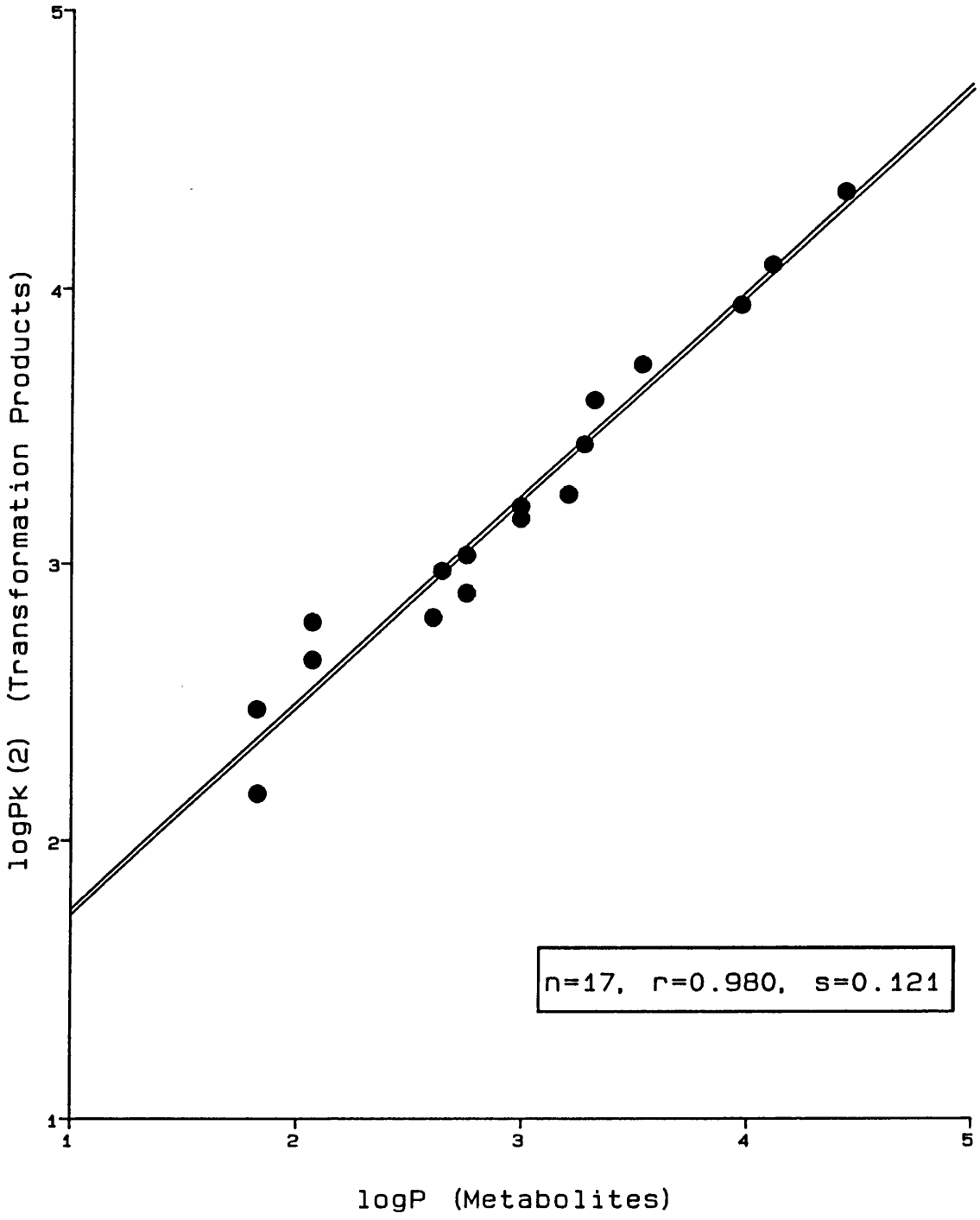


Fig.2.9

CHAPTER 3

ELUOTROPIC STRENGTH OF SOLVENTS:

Prediction and Application in the Reversed-Phase HPLC

3.1 INTRODUCTION

Reversed-phase HPLC is one of the most widely used analytical techniques. It is estimated[3.1] that 70-80% of all analytical separations are carried out using this technique. It is, therefore, not surprising that a considerable degree of interest exists in some fundamental aspects of this technique.

One area which has attracted much attention is solvent optimisation. The approach generally adopted for the prediction of optimum solvent composition for a specific separation problem is to chromatograph a given set of solutes using various combinations of solvents, and then to express the retentions by some quantitative parameter e.g. Chromatographic Optimisation Function (COF)[3.2]. Optimisation is sought by correlating COF with solvent composition. This is known as a mixture-design statistical(MDS) technique[3.2].

The first step, generally, is to find the composition of methanol-water which provides a chromatogram with capacity factors in the range of 1 to 10 or if required, 1 to 20. This is achieved by conventional "trial-and-error" methods or by predicting an isocratic composition from gradient runs[3.3]. The latter method, unfortunately, requires solvent-specific constants to be determined for each column which requires considerable experimental work.

The next step is to find compositions of acetonitrile-water and tetrahydrofuran-water giving similar chromatograms. In general, "trial-and-error" methods are used. However, it would be ideal if these compositions could be predicted.

In order to achieve this goal various workers have proposed different solvent "strength" parameters such as P'[3.4], S[3.5], $\delta(T)$[3.6] and also empirical transfer rules[3.3] for reversed-phase LC, whilst for adsorption LC (LSC), ϵ° has been proposed for alumina[3.7] and for carbon[3.8] stationary phases. Discussion of adsorption LC is outside the scope of this study. Research workers have made use of the parameters proposed for reversed-phase LC, although these parameters have some limitations and do not provide adequate flexibility in their routine use as desired by chromatographers.

In the present study the possibility of using the n-octanol/water partition coefficient of a solvent as a strength parameter has been explored and comparisons has been made with other parameters and also with an empirical approach.

3.2 THEORETICAL

In reversed-phase LC, retention of a solute is described mainly as a function of its solvophobic interaction [3.9-3.13]. Hence retention is explained as a consequence of partitioning, or is due to the forces involved in the partitioning interaction, of solute between stationary and mobile phase [3.14-3.16]. Oscik [3.17] was the first to derive an equation for LSC with mixed mobile phases which reflects a partition effect in the chromatographic process. Such effects are dominant in the typical reversed-phase system [3.14].

Whatever mechanism(s) may be responsible for the retention in RPLC, it is a fact that in this mode of chromatography, retention parameters are correlated to n-octanol/water partition coefficients (logP) of the solutes[3.18].

It is known that retention is a sensitive function of the quantitative (strength) and qualitative (selectivity) composition of the mobile-phase. However, it should be realised that whilst the strength of the mobile phase is a major factor in controlling the retention, in the majority of cases minor changes

in retention can be obtained by selectivity changes produced by employing iso-eluotropic solvents. This can be shown by a multisolvent space diagram (Fig.3.1). It can be seen that there are an infinite number of compositions which can be found in this space. However only those compositions which lie on the triangular plane(ABC) indicated are capable of producing chromatograms with retentions of all solutes within the desired limit of capacity factor. Therefore it is this triangular plane which is important for optimisation. Ideally, knowing the composition of A by empirical means, or other, we should be able to predict the quantitative composition of B and C.

3.2.1 Snyder's Polarity Index (P')

Snyder[3.19] proposed Polarity Index (P') as a chromatographic strength parameter. It was originally developed for GC and normal-phase LC solvents, but according to Snyder and Kirkland[3.20] and Glajch et al.[3.2] it can also be used for RPLC. Table 3.1 shows P' and other strength parameters for a few solvents widely used in RP-HPLC.

The solvent strength is inversely proportional to its P' index. This relationship suggests that THF is the strongest solvent in this group. However it also shows that acetonitrile is a weaker solvent than methanol, i.e. P'(ACN) is greater than P'(MeOH). In practice, generally, it is found that ACN is stronger than MeOH. Furthermore, methanol and acetone are equivalent in

their solvent strength (P'=5.1) which contradicts practical findings. This is shown more clearly by the comparison of experimental and predicted compositions using P' and other strength parameters in Table 3.2. This discrepancy cannot be resolved simply. Hence it seems that we need a better parameter to measure the chromatographic strength of the solvents in RPLC.

The experimentally found equivalent compositions given in Table 3.2 were used by the authors, of the given reference, mostly for solvent optimisation using the MDS technique. However, it should be noted that they are not strictly iso-eluotropic, as the retentions of the last peak are not similar, in fact in some cases they are significantly different. Therefore judgement about the equivalence should be made with due consideration.

3.2.2 Solvent Strength Parameter (S)

According to Snyder et al.[3.5] the retention (lnk') of a solute in RPLC is best approximated, within experimental errors, by following expression:

$$\ln k' = \ln k(0) - S \cdot \phi \qquad \ldots(\text{Eq.3.1})$$

where,
lnk(0) is the extrapolated value of lnk' when Ø is zero, assuming Eq.3.1 is true over the range $0<\phi<1$,
S is considered to be a solvent-strength parameter, and
Ø is the volume fraction of solvent in water.

This relationship clearly suggests that S is a coefficient whose value should be a constant for a given solvent.

However they pointed out that S is not a constant which is characteristic of a given solvent but varies, for unknown reasons, with other separation parameters and therefore suggested a definitive experimental study which was reported by Dolan et al.[3.28]. This study found that for 9 solutes the S value did not vary by more than ±10-20% for a given column and therefore the authors argued that little variation was to be expected in S for other solutes as well.

However S is not entirely independent of the nature of the solute as a relationship was observed between the number of carbon atoms in a homologous series and their retentions[3.28]. Similar observations were also made by Poile[3.29], Englehardt[3.30] and Jandera[3.31,3.32]. It was also shown[3.28] that S, for a given solute, was almost invariant with stationary phases, but did vary with the solvent type. Furthermore, Schoenmakers et al.[3.3] found that S was dependent on lnk(0) by the following equation and on the solvent type.

$$S = p + q \cdot \ln k(0) \qquad \ldots(\text{Eq.3.2})$$

In our analysis of their data[3.3] it was found that lnk(0) was related with the logP (n-octanol/water) of the solutes(n=27, r=0.943, s=0.284, F=200.3) for

MeOH-Water solvent systems. This result again confirms the view that S is dependent on the nature of the compounds under analysis. An examination of Table 3.1 reveals that when described by the S parameter, methanol and acetonitrile differ only marginally in their eluotropic strength. However, when used to predict acetonitrile compositions, produced values that were much greater than the experimental ones, as shown in Table 3.2. Therefore it seems that S cannot used as an ideal strength parameter.

3.2.3 Schoenmakers' Empirical Transfer Rules

Schoenmakers et al.[3.3] obtained transfer rules empirically from experimental data on 32 solutes from three different classes, viz. acidic, basic and neutral compounds. The transfer equations are given below:

$$\emptyset a = 0.57\emptyset m + 0.32\emptyset m^2 \qquad \text{...(Eq.3.3)}$$

$$\emptyset t = 0.66\emptyset m \qquad \text{...(Eq.3.4)}$$

Where,
m, a and t is for MeOH, ACN and THF respectively and
Ø is the volume fraction of solvent in water.

These rules are found to be useful, although no theoretical basis was proposed to explain them. However it is not possible to calculate equivalents for a system containing more than two solvents.

Furthermore similar rules should be established for solvents other than those given here. Compositions computed by Eq.3.3 and 3.4 were found to be in good agreement with the experimental results as shown in Table 3.2. Because these transfer rules are based upon experimental data, they predict that acetonitrile is stronger than methanol (Fig.3.3) which contradicts the predictions by P′ and S parameter. Although these transfer rules are practically useful they may not be applicable universally to other RPLC chromatographic systems for the reason that the experimental data were obtained using a single stationary-phase, which due to its characteristics may influence the coefficients of Eq.3.3 and Eq.3.4. Furthermore, obtaining similar transfer rules for various solvents would require considerable experimental effort especially if the data are collected from a variety of stationary-phases.

3.2.4 Solubility Parameter(δ(T))

Schoenmakers et al.[3.33] were of the opinion that some of the problems that might occur when a solubility parameter is used as a solvent strength parameter, were as follows.

1. Water behaves so uniquely that it is difficult to describe it in terms of δ(T),

2. Chemically bonded phases may not have the properties of bulk phases for which solubility theory has been derived, and

3. It is not possible to calculate the solubility parameter for mixed solvents especially when there are more than two solvents in the mixture.

However, the following expression was used for two solvent mixtures.

$$\delta(T,m) = \delta(T,p) - \emptyset\cdot\{\delta(T,p) - \delta(T,q)\} \quad \text{...(Eq.3.5)}$$

Where,
$\delta(T)$ is total solubility parameter,
m stands for mixture,
p for more polar solvent,
q for less polar solvent.

This is a linear relationship, which does not agree with the experimental results[3.34] where a non-linear relationship was shown. This means that the given expression should be considered only as an approximation and not an accurate way to calculate solvent strength.

Similar difficulties with the use of $\delta(T)$ were also reported earlier[3.6], namely that accurate retentions in LC could not be predicted using $\delta(T)$. Although the bulk partition behaviour of solutes may be described by $\delta(T)$ (n=7, r=0.874, s=0.615), it is not as precise as the method of Leo et al.[3.35] (n=7, r=0.950, s=0.287). In spite of these reservations, $\delta(T)$ is found to be a good predictor of the binary solvent compositions examined (see Table 3.2). However, the predictions are not in complete agreement with observations made by

Schoenmakers et al.[3.3] in their experimental study. For example, the ACN equivalent for MeOH showed a curvilinear relationship in the experimental results as compared to the linear relationship found in the predicted compositions by $\delta(T)$. Thus although $\delta(T)$ is a good predictor of solvent strength it does have its limitations.

3.2.5 Partition Coefficient (Ps)

The retention in RPLC, as mentioned earlier, is mainly hydrophobic in nature, and there are several reports (see reference 3.18 for a comprehensive list) showing good correlation between lnk and logP of solutes. It shows that the competition between the solute and the mobile phase for the same retention site i.e. the stationary phase, controls the retention.

Thus, it seems that the hydrophobic property of the solvent is also important for the solubility and/or elution of the solutes. Furthermore, the solubility of solutes/solvents is related to their partition coefficient and melting point as shown by Yalkowski et al.[3.36]

Further support for the use of a hydrophobic parameter such as logP is provided by the findings of Tanaka and Thornton[3.11], who showed that pure water does not appear to be unique with respect to its chromatographic properties as compared to methanol-like solvents. The only difference found was that water is at the end of the continuum of hydrophobicity. Karger et al.[3.10]

reported that the hydrophobic selectivity of different solvents is approximately independent of their chemical nature, which again supports the idea that logP could be used as an absolute strength parameter, provided the chromatographic strength shows good agreement with the experimental data.

With these views in mind it was considered that the partition coefficient(n-octanol/water) of a solvent could be used as a solvent strength parameter, which shall now be referred to as Ps, where s indicates solvent. The experimental logPs values for the four solvents, viz. water, methanol, acetonitrile and tetrahydrofuran, widely used in RPLC were obtained from the literature[3.37]. These values agreed well with theoretical logPs values, calculated according to the method of Hansch and Leo[3.37], as shown in Table 3.3.

For pure solvents, the partition coefficient can be correlated with solvent strength. However, for mixtures of solvents the partition coefficient of each solvent may be added according to their proportions i.e. their mole fractions multiplied by logP. This assumption is made on the basis that when an analyte interacts non-specifically with the surrounding constituent molecules of the mobile-phase, the probability of interaction is proportional to their molar proportion. This can be expressed mathematically as follows.

$$\log Ps(m) = \sum_{i=1}^{n} X(i) \cdot \log Ps(i) \qquad \ldots(Eq.3.6)$$

Where,

m indicates mixture,

X(i); the mole fraction of the ith solvent,

logPs(i); the n-octanol/water logP for ith solvent, and

n; the total number of solvents used in the mixture.

The proposed method (Eq.3.6) was used to calculate logPs(m) for solvent-water mixtures of the solvents listed in Table 3.1. The number of moles of each solvent in the mobile-phases were computed from their volumes using density and molecular weight data given in Table 3.4. A graph of the computed strength parameter, logPs(m), of different solvent-water mixtures is shown in Fig.3.2. It is apparent that the calculated strength differ marginally at higher percent of the solvent in the mixture. Interestingly there seem to be four groups of the solvent as they show similar patterns of the strength curves, especially in the case of acetonitrile and ethanol. This is not surprising as the mol. wt. and the logP values of these two solvents are very close.The groups are:

1. Methanol,

2. Acetonitrile and Ethanol,

3. Acetone and Dioxane, and

4. Tetrahydrofuran and i-Propanol.

Groups 2 and 3 are showing similar pattern of the strength curves and at about 90% of the solvent in the mixture they show almost identical calculated strength.

Theoretically, all compositions with the same logPs(m) value are iso-eluotropic. Based on this assumption calculations were made to find the compositions of solvent-water mixtures which are iso-eluotropic with respect to methanol-water. This involved calculation of the logPs(m) for the given mixture e.g. 50% MeOH, and then the volume composition of the desired solvent mixture was derived e.g. X% ACN or Y% THF in water, using a modified linear interpolation algorithm[3.38].

This is an iterative interpolation process where the values of X or Y are constantly changed until the logPs(m) value of these compositions matches(tolerance used for the match used was $\pm$10E-4) with the logPs(m) of the given composition, in this case 50% MeOH. This numerical method was found to be fast and provided the accuracy required. A listing of the programme, "STRENGTH", used for solvent strength calculation is given in Appendix A.

Fig.3.3 shows solvent-water compositions iso-eluotropic to methanol-water for solvents listed in Table 3.1 and a list of solvent strength, logPs, and corresponding compositions is given in Table 3.5. An examination of Fig.3.3 confirms observations noted earlier that some of the solvents are comparable in their solvent strength. In this figure similar pattern is observed.

From this data, as presented in Table 3.5, transfer equations similar to Schoenmakers' rules, were obtained. These equations are given below. Similar rules were also developed for other solvents and the

coefficients of quadratic relationships are given in Table 3.6.

1. This study :

$$Va = 0.698\ Vm + 0.00081\ Vm^2 \qquad \text{...(Eq.3.7)}$$

$$Vt = 0.621\ Vm + 0.00046\ Vm^2 \qquad \text{...(Eq.3.8)}$$

2. Schoenmakers':

$$Va = 0.570\ Vm + 0.00320\ Vm^2 \qquad \text{...(Eq.3.3a)}$$

$$Vt = 0.660\ Vm \qquad \text{...(Eq.3.4a)}$$

Where,

V = volume% and

m,a and t indicates MeOH,ACN and THF respectively.

Equations for ACN and THF are compared graphically with Schoenmakers' rules in Fig.3.4. An examination of Fig.3.4 suggests that Schoenmakers' empirically obtained transfer rules (Eq.3.3a and 3.4a) based on experimental data, compare very well with the rules (Eq.3.7 and 3.8) derived on the theoretical basis proposed in this study. The relationship between Vt and Vm could be described in a linear form without losing statistical significance, however, the quadratic relationship was retained for better accuracy. Although the curve for ACN shows some departure from the empirical rule at higher methanol% in water, the predicted compositions for experimental data seem to correlate very well, as shown in Table 3.2. Statistical analysis of data in Table 3.2 is given in

Table 3.7. It is apparent from this analysis that parameter logPs provides the highest coefficient of determination (r^2=80.4%) between experimental and predicted solvent compositions with the lowest standard deviation. It is also obvious that Schoenmakers' empirical transfer rules compare well, statistically, with the logPs approach.

Discussions so far have shown how logPs compares well with other parameters. In order to further check the validity of the predicted compositions by the logPs approach, experiments were conducted for simple un-ionizable compounds using RPLC.

3.3 EXPERIMENTAL

Chromatographic studies were performed using a Spectra-Physics model SP8100 liquid chromatograph with a UV-Vis. detector, model SP8440 and a computing integrator, model SP4200, and a stainless-steel column, 150x4.6 mm I.D., packed with 6μm Zorbax-ODS (Du Pont). Methanol, acetonitrile and tetrahydrofuran were HPLC grade(Fisons,UK). Glass distilled deionized water was used to prepare phosphate buffer(0.0025M) to maintain pH at 6.9 and is referred in this study as water. Samples were dissolved in a mixture of MeOH-Water. All injections were made by autoinjecter. All the analyses were carried out at 35°C in a hot air oven, and detection was monitored at 254nm. Data analysis and computing was carried out on a Honeywell 68 DPS level 2 via RJE Honeywell Level 6/43 using MINITAB software or

in FORTRAN77 and in BASIC using a microcomputer (SinclairQL, Sinclair Research Ltd.,UK) with 128K RAM. A mixture was prepared containing benzonitrile(C1), benzene(C2), toluene(C3),naphthalene(C4) and biphenyl(C5). This mixture was analysed in triplicate by isocratic mode using mobile-phase systems containing 70, 60 and 50% MeOH in water and their predicted equivalents for ACN and THF, which are shown in Table 3.8, together with their logPs(m) values.

3.4 RESULTS AND DISCUSSION

Detailed examination of Table 3.8 reveals that, within the k' range of 1 to 10 , iso-eluotropic compositions i.e. those compositions with identical logPs(m) values produced similar retention values, especially for compounds C1-C3. However naphthalene(C4) and biphenyl(C5) gave lower retentions than expected when in THF-water mixtures. This suggests that probably the selectivity of THF towards C4 and C5 is different from that of methanol and acetonitrile. It may be that the cyclic structure of THF permits better stacking with C4 and C5 during the solvation process, which would reduce retention. Nevertheless, as mentioned earlier, the compositions predicted using logPs are well supported by Schoenmakers' transfer rules, which were obtained from data on 32 solutes. Hence it is expected that predictions made by the logPs parameter should be generally applicable.

3.5 CONCLUSIONS

The logarithm of Partition Coefficient(n-Octanol/Water) of a solvent, logPs, could be used as a strength parameter, because its use has resulted in relatively precise predictions of iso-eluotropic compositions and it offers many advantages over the other parameters discussed.

1. Iso-eluotropic compositions predicted by logPs are in good agreement with those predicted by Schoenmakers' transfer rules, based upon experimental data from 32 solutes.

2. Experimentally, iso-eluotropic compositions predicted by logPs gave good agreement between methanol and acetonitrile for five solutes. Tetrahydrofuran showed expected retentions of C1-C3 but the selectivity towards two solutes was different as compared to methanol and acetonitrile.

3. The values of logPs for any solvent are easily available from the literature or can be calculated theoretically, or can be determined experimentally, unlike other parameters which requires either extensive chromatographic analysis or detailed calculations using basic molecular properties.

4. It is possible to calculate the strength of a mixture of solvents, using logPs, and to find another iso-eluotropic mixture of desired solvents which is of considerable value in optimisation procedures.

TABLE 3.1

STRENGTH PARAMETERS FOR THE SOLVENTS USED IN RPLC

	Strength Parameter			
Solvent	P'[3.4]	S[3.5]	δT[3.6]	logPs*
Water	10.2	0.0	25.52	-1.38
Methanol	5.1	3.0	15.85	-0.82**
Acetonitrile	5.8	3.1	13.15	-0.34
Ethanol	4.3	3.6	13.65	-0.31
Dioxane	4.8	3.5	10.65	-0.27
Acetone	5.1	3.4	10.51	-0.24
Propan-2-ol	3.9	4.2	12.37	+0.30
Tetrahydrofuran	4.0	4.4	9.88	+0.46

* n-Octanol-water logP of solvents[3.37]

** Although several values were available this value was chosen as it gave satisfactory predictions.

TABLE 3.2

COMPARISON OF THE COMPOSITIONS PREDICTED BY DIFFERENT APPROACHES

Solvent	Expt.	Snyder		Schoenmakers			Last peak* retention
	%v/v	P' [3.4]	S [3.5]	Emp.¤ [3.3]	δT [3.6]	logPs	
MeOH[3.2]	63	63.0	63.0	63.0	63.0	63.0	7.8 k'
ACN	52	73.0	61.0	48.6	49.2	46.7	7.9
THF	39	51.8	43.0	41.6	38.9	41.0	6.7
MeOH[3.21]	50	50.0	50.0	50.0	50.0	50.0	35.8 k'
ACN	40	58.0	48.4	36.5	39.1	36.6	17.3
THF	37	41.1	34.1	33.0	30.9	32.2	10.3
MeOH[3.22]	60	60.0	60.0	60.0	60.0	60.0	15.0 tR
ACN	40	69.6	58.1	45.7	46.9	44.3	23.0
THF	30	49.4	40.9	39.6	37.1	38.9	24.0
MeOH[3.23]	35	35.0	35.0	35.0	35.0	35.0	39.0 tR
ACN	20	40.6	33.9	23.9	27.4	25.2	43.0
THF	12	28.8	23.9	23.1	21.6	22.3	57.0
MeOH[3.24]	41	41.0	41.0	41.0	41.0	41.0	6.2 cm
ACN	30	47.5	39.7	28.7	32.1	29.7	5.5
THF	28	33.7	27.9	27.0	25.3	26.2	6.2
MeOH[3.25]	65	65.0	65.0	65.0	65.0	65.0	5.9 cm
ACN	50	75.3	62.9	50.6	50.8	48.3	7.0
THF	45	53.5	44.3	42.9	40.2	42.3	6.4
MeOH[3.26]	50	50.0	50.0	50.0	50.0	50.0	11.6 tR
ACN	32	58.0	48.4	36.5	39.1	36.6	12.6
THF	33	41.1	34.1	33.0	30.9	32.2	13.0
MeOH[3.27]	50	50.0	50.0	50.0	50.0	50.0	13.9 tR
ACN	37	58.0	48.4	36.5	39.1	36.6	14.0
THF	32	41.1	34.1	33.0	30.9	32.2	14.0
MeOH**		100.0	100.0	100.0	100.0	100.0	
ACN		115.9	96.8	89.0	78.2	77.1	
THF		82.3	68.2	66.0	61.8	66.8	

¤ Empirical Transfer Rules

* Retention of last peak in terms of capacity factor (k'), retention time (tR) or distance from injection(cm).

** Equivalent for 100% methanol (calculated).

Note: (1) %v/v of solvent in Water/Bufffer.

(2) Values in square brackets are references.

TABLE 3.3

THEORETICAL AND EXPERIMENTAL logP VALUES OF SOLVENTS

Solvent	logP	
	Theoretical	Experimental*
Water	-1.41	-1.38
Methanol	-0.75	-0.82
Acetonitrile	-0.38	-0.34
THF	+0.46	+0.46

* Obtained from Ref.[3.37]

TABLE 3.4
MOLECULAR WEIGHT, DENSITY AND logP VALUES OF SOLVENTS USED IN RP-HPLC.

Solvent	Code	Mol.Wt.§ amu	Density§ (g/ml)	logP¤
Water	H2O	18.00	1.0000	-1.38
Methanol	MeOH	32.04	0.7928	-0.82
Acetonitrile	ACN	41.05	0.7871	-0.34
Ethanol	EtOH	46.07	0.7893	-0.31
Dioxane	DXN	88.12	1.0342	-0.27
Acetone	Me2CO	58.08	0.7899	-0.24
Propan-2-ol	PrOH	60.11	0.7855	+0.30
Tetrahydrofuran	THF	72.01	0.8719	+0.46

§ Data at 20°C [3.39]
¤ Data from ref.[3.37]

TABLE 3.5
SOLVENT-WATER MIXTURES ISO-ELUOTROPIC TO METHANOL-WATER MIXTURE

logPs	MeOH%*	ACN%	THF%	Me2CO%	PrOH%	DXN%	EtOH%
-1.380	0.00	0.00	0.00	0.00	0.00	0.00	0.00
-1.377	1.00	0.70	0.62	0.89	0.63	1.06	0.76
-1.375	2.00	1.39	1.25	1.78	1.26	2.12	1.51
-1.372	3.00	2.09	1.87	2.67	1.90	3.17	2.27
-1.370	4.00	2.79	2.50	3.56	2.53	4.21	3.03
-1.367	5.00	3.49	3.12	4.44	3.17	5.25	3.79
-1.365	6.00	4.19	3.75	5.32	3.80	6.28	4.54
-1.362	7.00	4.90	4.38	6.20	4.44	7.30	5.30
-1.359	8.00	5.60	5.01	7.08	5.08	8.32	6.06
-1.356	9.00	6.31	5.64	7.96	5.72	9.34	6.82
-1.354	10.00	7.02	6.27	8.83	6.36	10.35	7.58
-1.351	11.00	7.73	6.90	9.70	7.00	11.35	8.34
-1.348	12.00	8.44	7.53	10.57	7.64	12.35	9.11
-1.345	13.00	9.15	8.17	11.44	8.28	13.34	9.87
-1.342	14.00	9.87	8.80	12.30	8.93	14.33	10.63
-1.339	15.00	10.58	9.44	13.17	9.57	15.31	11.39
-1.336	16.00	11.30	10.07	14.03	10.22	16.28	12.16
-1.333	17.00	12.02	10.71	14.89	10.86	17.26	12.92
-1.330	18.00	12.74	11.35	15.75	11.51	18.22	13.68
-1.327	19.00	13.46	11.99	16.60	12.16	19.18	14.45
-1.324	20.00	14.18	12.62	17.45	12.81	20.14	15.21
-1.321	21.00	14.90	13.27	18.31	13.46	21.09	15.98
-1.318	22.00	15.63	13.91	19.15	14.11	22.03	16.75
-1.314	23.00	16.36	14.55	20.00	14.76	22.97	17.51
-1.311	24.00	17.09	15.19	20.85	15.41	23.91	18.28
-1.308	25.00	17.82	15.84	21.69	16.07	24.84	19.05
-1.304	26.00	18.55	16.48	22.53	16.72	25.76	19.82
-1.301	27.00	19.28	17.13	23.37	17.38	26.68	20.59
-1.297	28.00	20.01	17.77	24.21	18.04	27.60	21.35
-1.294	29.00	20.75	18.42	25.04	18.69	28.51	22.12
-1.290	30.00	21.49	19.07	25.88	19.35	29.41	22.89
-1.287	31.00	22.23	19.72	26.71	20.01	30.31	23.67
-1.283	32.00	22.97	20.37	27.54	20.67	31.21	24.44
-1.279	33.00	23.71	21.02	28.37	21.33	32.10	25.21
-1.275	34.00	24.45	21.67	29.19	22.00	32.99	25.98
-1.272	35.00	25.20	22.32	30.02	22.66	33.87	26.75
-1.268	36.00	25.95	22.98	30.84	23.33	34.75	27.53
-1.264	37.00	26.69	23.63	31.66	23.99	35.62	28.30
-1.260	38.00	27.44	24.29	32.48	24.66	36.49	29.07
-1.256	39.00	28.19	24.94	33.29	25.33	37.35	29.85
-1.252	40.00	28.95	25.60	34.11	25.99	38.21	30.62
-1.248	41.00	29.70	26.26	34.92	26.66	39.07	31.40
-1.243	42.00	30.46	26.92	35.73	27.33	39.92	32.18
-1.239	43.00	31.22	27.58	36.54	28.01	40.77	32.95
-1.235	44.00	31.98	28.24	37.35	28.68	41.61	33.73
-1.230	45.00	32.74	28.90	38.15	29.35	42.45	34.51
-1.226	46.00	33.50	29.56	38.96	30.03	43.28	35.29
-1.221	47.00	34.26	30.23	39.76	30.70	44.11	36.07
-1.217	48.00	35.03	30.89	40.56	31.38	44.94	36.85
-1.212	49.00	35.80	31.56	41.35	32.06	45.76	37.62
-1.207	50.00	36.56	32.22	42.15	32.74	46.57	38.40

* Refer Table 3.4 for solvent codes

TABLE 3.5 (Continued)
SOLVENT-WATER MIXTURES ISO-ELUOTROPIC TO METHANOL-WATER MIXTURE

logPs	MeOH%*	ACN%	THF%	Me2CO%	PrOH%	DXN%	EtOH%
-1.203	51.00	37.33	32.89	42.94	33.42	47.39	39.19
-1.198	52.00	38.11	33.56	43.74	34.10	48.20	39.97
-1.193	53.00	38.88	34.23	44.53	34.78	49.00	40.75
-1.188	54.00	39.66	34.90	45.32	35.46	49.80	41.53
-1.183	55.00	40.43	35.57	46.10	36.14	50.60	42.31
-1.177	56.00	41.21	36.24	46.89	36.83	51.40	43.10
-1.172	57.00	41.99	36.91	47.67	37.51	52.18	43.88
-1.167	58.00	42.77	37.59	48.45	38.20	52.97	44.67
-1.161	59.00	43.56	38.26	49.23	38.89	53.75	45.45
-1.156	60.00	44.34	38.94	50.01	39.58	54.53	46.24
-1.150	61.00	45.13	39.62	50.79	40.27	55.30	47.02
-1.144	62.00	45.92	40.29	51.56	40.96	56.08	47.81
-1.138	63.00	46.71	40.97	52.34	41.65	56.84	48.59
-1.133	64.00	47.50	41.65	53.11	42.34	57.61	49.38
-1.126	65.00	48.29	42.33	53.88	43.04	58.36	50.17
-1.120	66.00	49.09	43.02	54.65	43.73	59.12	50.96
-1.114	67.00	49.88	43.70	55.41	44.43	59.87	51.75
-1.108	68.00	50.68	44.38	56.18	45.13	60.62	52.54
-1.101	69.00	51.48	45.07	56.94	45.82	61.37	53.33
-1.095	70.00	52.29	45.75	57.70	46.52	62.11	54.12
-1.088	71.00	53.09	46.44	58.46	47.22	62.85	54.91
-1.081	72.00	53.89	47.12	59.22	47.93	63.58	55.70
-1.074	73.00	54.70	47.81	59.97	48.63	64.31	56.49
-1.067	74.00	55.51	48.50	60.73	49.33	65.04	57.28
-1.060	75.00	56.32	49.19	61.48	50.04	65.76	58.08
-1.052	76.00	57.13	49.88	62.23	50.74	66.48	58.87
-1.045	77.00	57.95	50.58	62.98	51.45	67.20	59.66
-1.037	78.00	58.76	51.27	63.73	52.16	67.92	60.46
-1.029	79.00	59.58	51.96	64.48	52.87	68.63	61.25
-1.021	80.00	60.40	52.66	65.22	53.58	69.33	62.05
-1.013	81.00	61.22	53.36	65.96	54.29	70.04	62.85
-1.005	82.00	62.05	54.05	66.70	55.00	70.74	63.64
-0.996	83.00	62.87	54.75	67.44	55.71	71.44	64.44
-0.988	84.00	63.70	55.45	68.18	56.43	72.13	65.24
-0.979	85.00	64.52	56.15	68.92	57.14	72.82	66.04
-0.970	86.00	65.36	56.85	69.65	57.86	73.51	66.84
-0.961	87.00	66.19	57.55	70.39	58.58	74.20	67.63
-0.951	88.00	67.02	58.26	71.12	59.29	74.88	68.43
-0.942	89.00	67.86	58.96	71.85	60.01	75.56	69.23
-0.932	90.00	68.69	59.67	72.58	60.74	76.23	70.04
-0.922	91.00	69.53	60.37	73.30	61.46	76.90	70.84
-0.911	92.00	70.37	61.08	74.03	62.18	77.57	71.64
-0.901	93.00	71.22	61.79	74.75	62.91	78.24	72.44
-0.890	94.00	72.06	62.50	75.47	63.63	78.90	73.24
-0.879	95.00	72.91	63.21	76.19	64.36	79.56	74.05
-0.868	96.00	73.76	63.92	76.91	65.08	80.22	74.85
-0.856	97.00	74.61	64.63	77.63	65.81	80.88	75.66
-0.845	98.00	75.46	65.35	78.35	66.54	81.53	76.46
-0.832	99.00	76.31	66.06	79.06	67.27	82.18	77.27
-0.820	100.00	77.17	66.78	79.77	68.01	82.82	78.07

* Refer Table 3.4 for solvent codes

TABLE 3.6

COEFFICIENTS FOR THE TRANSFER RULES FOR CONVERTING METHANOL-WATER TO SOLVENT-WATER COMPOSITION

Solvent	Code	Coefficients*	
		A	B
Acetonitrile	ACN	0.698	0.00081
Ethanol	EtOH	0.756	0.00025
Dioxane	DXN	1.040	-0.00215
Acetone	Me2CO	0.889	-0.00092
Propan-2-ol	PrOH	0.630	0.00050
Tetrahydrofuran	THF	0.621	0.00046

*; A and B are coefficient of general equation

$Vs = A \cdot Vm + B \cdot Vm^2$

Where,

V; Volume% of solvent

m; indicates methanol

and s; indicates solvent

TABLE 3.7

COMPARISON OF REGRESSION COEFFICIENTS AND CORRELATION BETWEEN OBSERVED AND PREDICTED COMPOSITIONS*

Predictor	Coefficients**		Std.Dev.	r^2%
	A	B		
S	3.32	0.738	5.938	66.1
P'	2.90	0.622	5.885	66.7
δT	-3.99	1.070	4.997	76.0
Empirical	-5.42	1.110	4.572	79.9
logPs	-7.93	1.200	4.517	80.4

* Data from Table 3.2

** Experimental = A + B·Calculated

TABLE 3.8

MEAN RETENTIONS*(k') OF FIVE COMPOUNDS UNDER SIMILAR ELUOTROPIC CONDITIONS

logPs(m)	Solvent	Compounds**				
		C1	C2	C3	C4	C5
	70.0%MeOH	1.87	3.44	5.35	7.58	12.13
-1.0946	52.3%ACN	1.98	3.54	5.42	7.46	11.12
	45.8%THF	1.89	3.75	4.96	(4.96)¶	(6.44)
	60.0%MeOH	2.62	5.33	9.62	15.62	29.40
-1.1557	44.3%ACN	3.21	5.94	10.02	15.21	25.58
	38.9%THF	2.83	6.19	8.94	(9.67)	(13.98)
	50.0%MeOH	4.15	8.85	18.31	35.50	78.04
-1.2074	36.6%ACN	4.83	9.40	17.54	30.15	57.60
	32.2%THF	4.15	10.15	16.62	(20.42)	(33.40)

* Average of three analysis.
** For C1-C5 see text.
¶ Values in parentheses are for those compounds for which THF probably shows different selectivity.

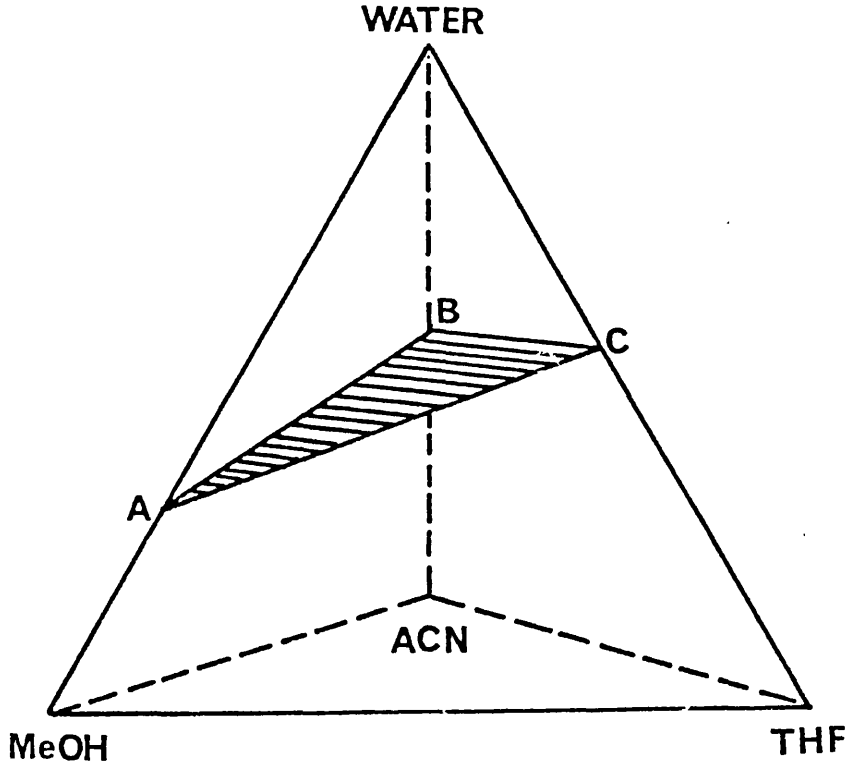


Fig.3.1 Multisolvent space showing a hypothetical iso-eluotropic plane.

PLOT OF SOLVENT STRENGTH, logPs(m), OF
DIFFERENT SOLVENT-WATER MIXTURES

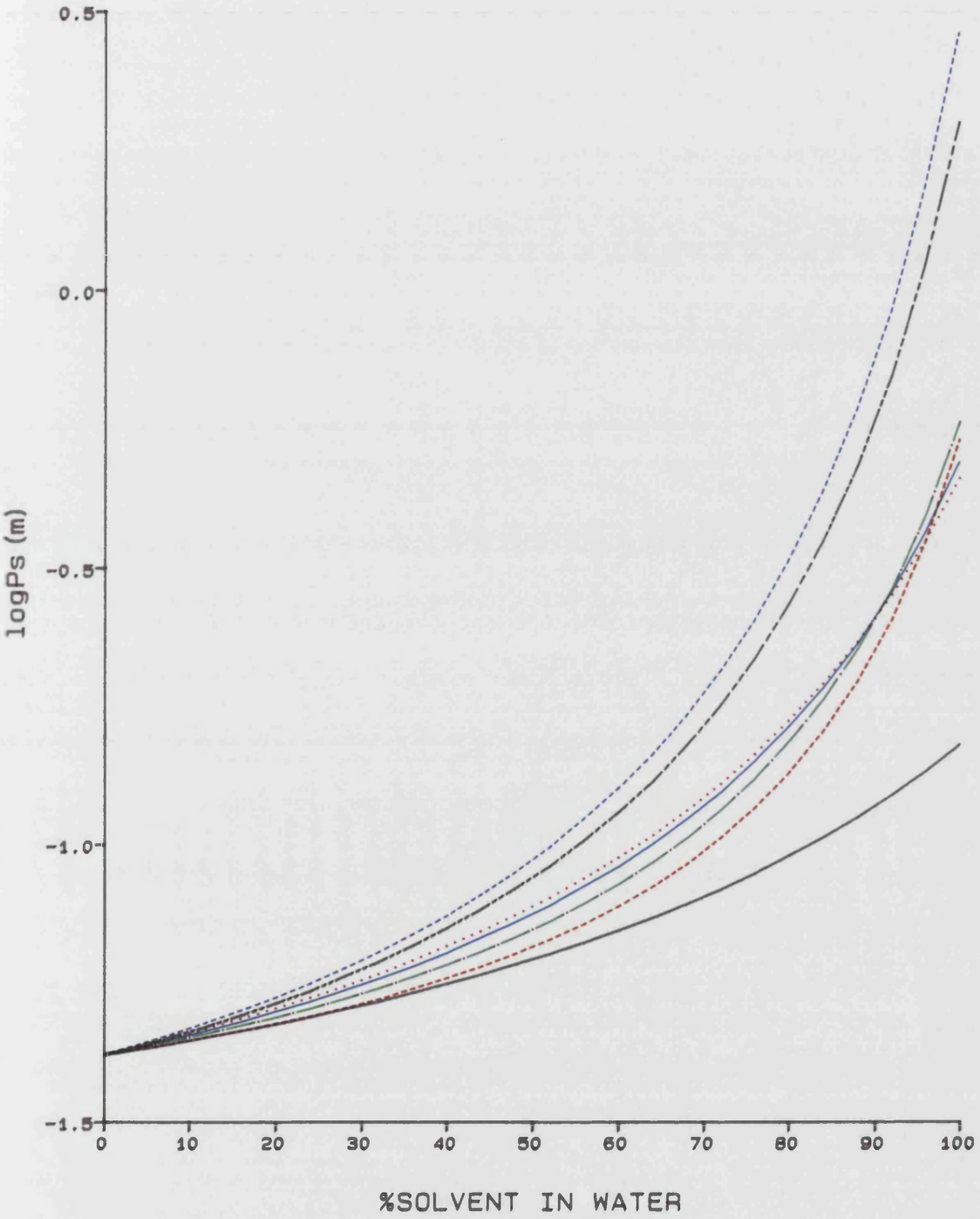
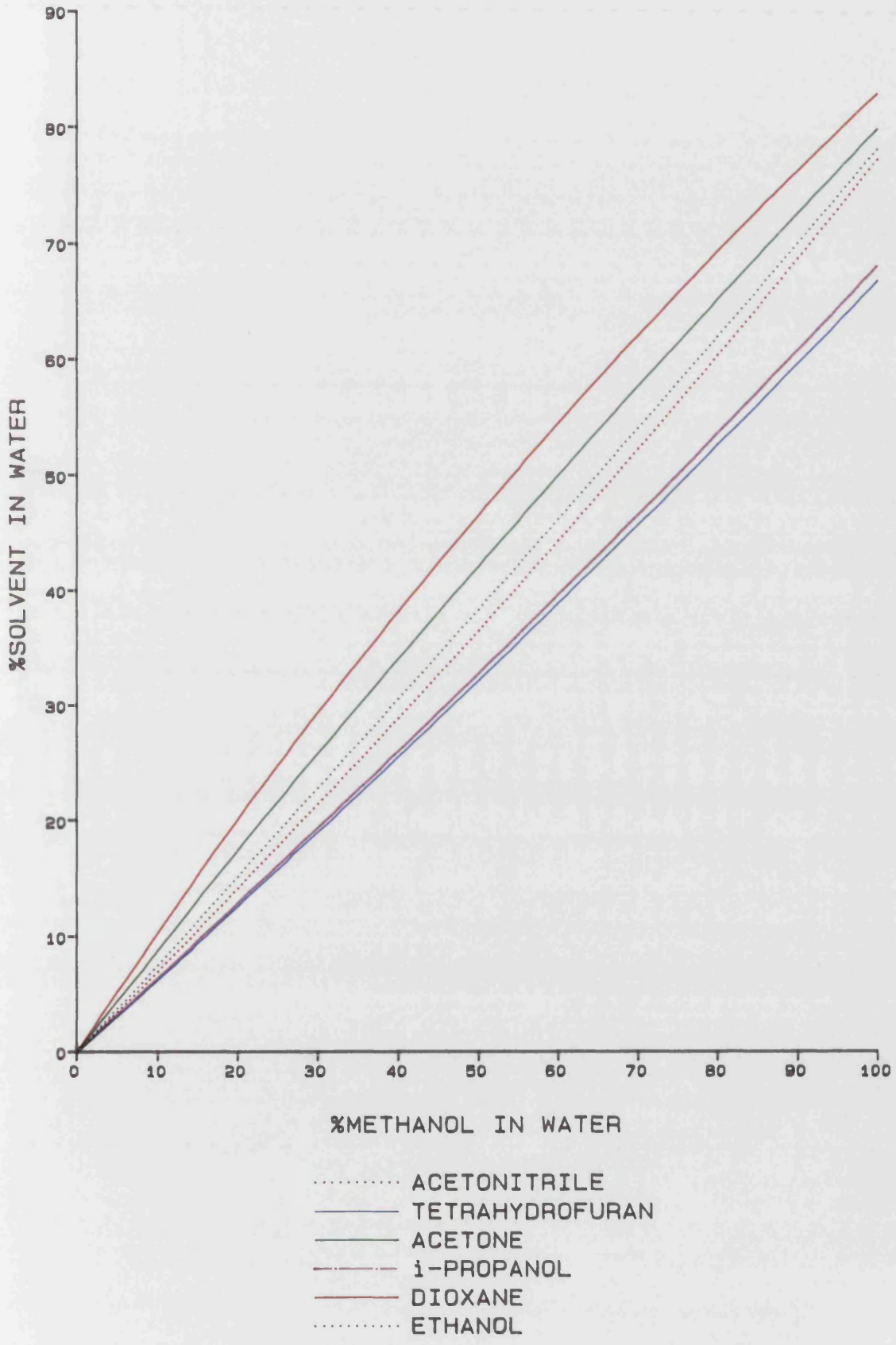
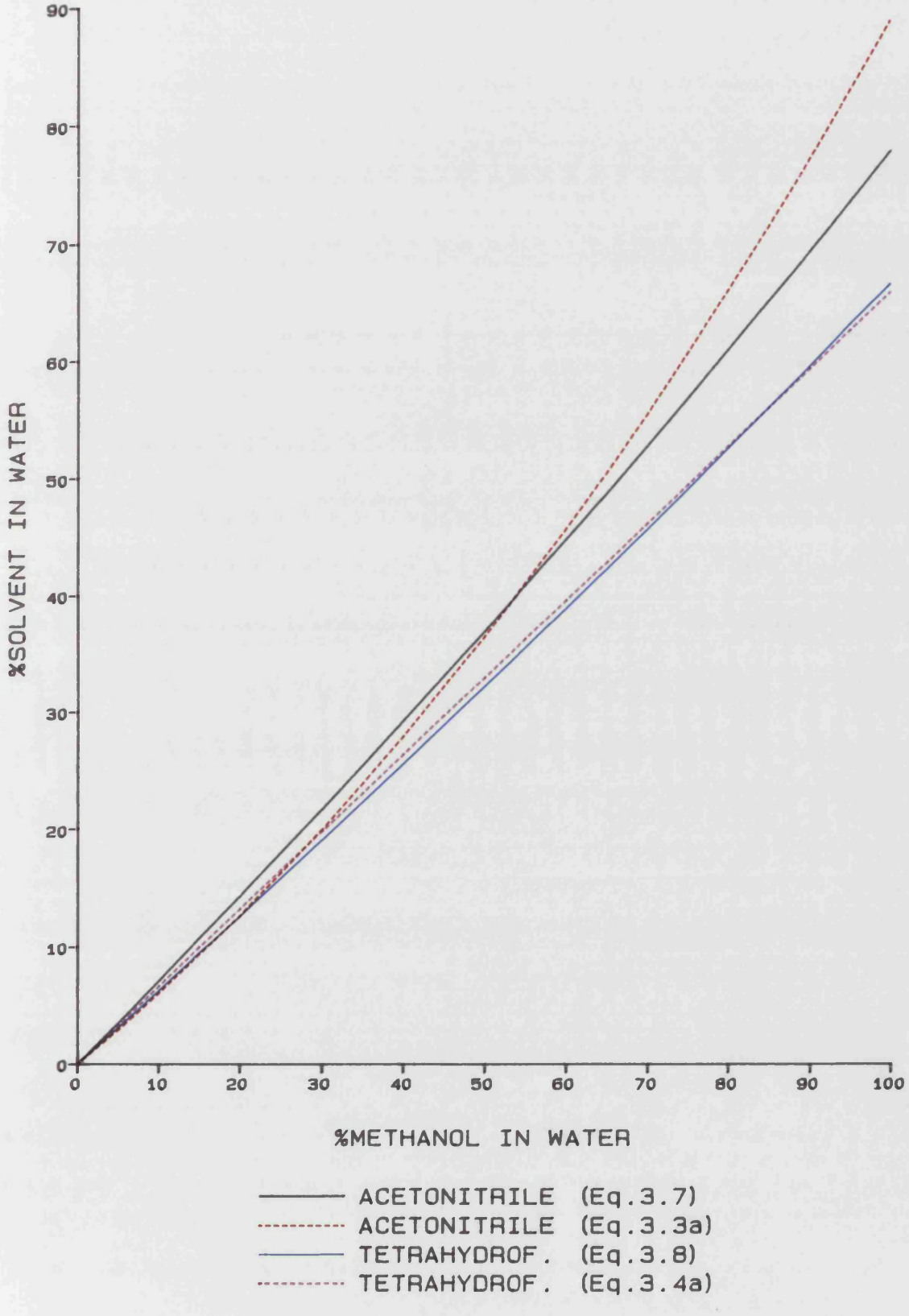


Fig.3.2

SOLVENT-WATER COMPOSITIONS ISO-ELUOTROPIC TO METHANOL-WATER AS PREDICTED BY logPs(m) PARAMETER



COMPARISON OF SCHOENMAKERS' TRANSFER RULES WITH THE EQUATIONS DEVELOPED IN THIS STUDY FOR ACN-WATER AND THF-WATER MIXTURES ISO-ELUOTROPIC TO MeOH-WATER MIXTURES



CHAPTER 4

CHROMATOGRAPHIC BEHAVIOUR OF SOLUTES IN REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

4.1 INTRODUCTION

In general, the accurate prediction of the conditions required in reversed-phase high performance liquid chromatography (RP-HPLC), for the adequate resolution of a particular group of analytes, remains an elusive goal for most chromatographers.

The most common approach is firstly, to select a column packing material and column dimension on the basis of previous experience, literature methods, and the recommendations from column manufacturers. The second step is the investigation and modification of the mobile phase by experimental "trial-and-error" in order to optimise the separation for resolution and speed.

Ideally, it should be possible to calculate the retention parameter of a given analyte in the chosen column - mobile phase system from the physico-chemical properties of the analyte, mobile phase and column. In practice, this has not yet been achieved and the development of chromatographic methods is usually a

difficult and time-consuming process.

Recently, the selection of the initial mobile phase composition has been improved by the introduction of systematic, rather than "trial-and-error" processes. These approaches include the use of a gradient run in order to select an isocratic composition[4.1], an iterative method[4.2], and an automated systematic "trial-and-error" type algorithm (simplex)[4.3,4.4]. These methods do not require a priori knowledge of the physico-chemical properties of the solutes.

Other workers have approached the problem by considering the physico-chemical properties of the analytes and using semi-deterministic or semi-empirical techniques, generally known as quantitative structure retention relationships (QSRR)[4.5-4.10]. This type of modelling is commonly based on the principles of linear free energy relationship in association with chromatographic theory, as used in quantitative structure activity relationships (QSAR) in drug design.

Commonly used parameters for QSRR type of studies are π(hydrophobic parameter), P(partition coefficient), F(correlation factor), χ(molecular connectivity index), L/B(shape parameter), and Vw (van der Waals volume).

The aim of this part of the study is to examine whether the partition coefficients of the analytes and mobile phases may be used to calculate the retention parameter of any un-ionized analyte in any mobile phase system.

4.2 THEORETICAL

Many physico-chemical parameters of a solute influence its retention in reversed-phase high performence liquid chromatography(RP-HPLC) and some may be estimated from the retention parameter[4.11]. However it has been suggested that in RP-HPLC the interaction between solute and hydrocarbonaceous stationary phase is the major factor of retention in the absence of organic modifiers[4.12], i.e., the process is governed by the so-called hydrophobic effect[4.13].

The hydrophobic interaction is the result of net repulsion between the water and non-polar moieties (of stationary phase and analyte). This theory had also been employed to describe the effect of solvents on certain chemical events[4.14,4.15]. This general theory does not restrict a solvophobic effect to aqueous media. However the very high cohesive density of water is responsible for the hydrophobic effect which is the most pronounced solvophobic effect.

It has been observed[4.16] that water is not an unique solvent in terms of its chromatographic property and that the hydrophobic selectivity of different solvents is approximately independent of their chemical nature[4.17]. Therefore, it is apparent that in the ion-suppressed (i.e. unionised analytes under solvent conditions employed) mode of RP-HPLC, the solvophobic effect could be considered as the most influential parameter.

The mathematical expressions like Eq.4.1 and 4.2 have been used to describe the retention of solute(s) in RP-HPLC[4.11,4.18].

$$\ln k' = A + B \cdot \log P \qquad \text{.....(Eq.4.1)}$$

$$\ln k' = A + B \cdot \phi + C \cdot \phi^2 \qquad \text{.....(Eq.4.2)}$$

Where,

k'= solute capacity factor,

logP= logarithm of Partition coefficient of solute,

ϕ= volume fraction of solvent in water, and

A,B and C; system dependent coefficients.

These equations are applicable under certain conditions only, i.e., it is not possible to combine retention data and solvent composition parameters for the same compound, for a given column, when analysed by different solvents or mixtures of solvents. Futhermore a general expression could be useful to describe retention behaviour of a mixture of solutes analysed under different isocratic solvent systems of different compositions.

It is apparent that a general model cannot be obtained unless a quantitative parameter for the eluotropic strength of the mobile phase is used instead of ϕ.

The significance and usefulness of logPs, logarithm of n-octanol/water partition coefficient of the solvent as solvent strength parameter[4.19] in RPLC has been discussed in Chapter 3. Table 3.1 lists logPs values

for the most frequently used organic modifiers employed in reversed-phase HPLC, and is compared with other "strength" parameters.

Since logPs can be measured for any solvent and offers advantages over other parameters it became the obvious choice for replacing the volume fraction term(Ø).

The logPs parameter could therefore be used to replace Ø in Eq.4.2 and to develop a model based on the hydrophobic parameters for solute and solvent viz., logP and logPs, respectively. As a first step the relationship between Ø and logPs was studied for three solvents, namely methanol, acetonitrile and tetrahydrofuran. It was found that a highly significant (Table 4.1) linear relationship (Eq.4.3) exists between Ø and 1/Ps, namely

$$Ø = A + B \cdot 1/Ps \qquad .....(Eq.4.3)$$

Where, Ps is the calculated n-octanol/water partition coefficient of the mobile phase.

It should be noted that although the value of coefficient B is negative (Table 4.1) this does not diminish the significance of the relationship between Ø and 1/Ps. Therefore Ø in Eq.4.2 can be substituted by 1/Ps which on rearrangement leads to Eq.4.4.

$$\ln k' = A + B \cdot 1/Ps + C \cdot 1/Ps^2 \qquad .....(Eq.4.4)$$

Eq.4.4 can be considered to be a general expression of

the retention of a given solute in any mixture of the solvents. A further step would be to obtain a relationship which is not only valid in the solvent domain but also in the solute domain.

Therefore in order to obtain a general model the relationships given by Eq.4.1 and Eq.4.4 need to be combined. The simplest combination is linear and provides Eq.4.5. Similar equations (Eq.4.6 to 4.10) containing interaction term(s) between logP and 1/Ps were also considered and subjected to statistical analysis(Table 4.2 and Table 4.3) for four sets of experimental data, viz., Data Set 1[4.20], Data Set 2[4.21], Data Set 3 and Data Set 4.

$$\ln k' = A + B \cdot \log P + C \cdot 1/Ps + D \cdot 1/Ps^2 \qquad \text{.....(Eq.4.5)}$$

$$\ln k' = A + B \cdot \log P + C \cdot (\log P)/Ps + D \cdot 1/Ps + E \cdot 1/Ps^2 \qquad \text{.....(Eq.4.6)}$$

$$\ln k' = A + B \cdot (\log P)/Ps + C \cdot 1/Ps^2 \qquad \text{.....(Eq.4.7)}$$

$$\ln k' = A + B \cdot (\log P)/Ps + C \cdot 1/Ps + D \cdot 1/Ps^2 \qquad \text{.....(Eq.4.8)}$$

$$\ln k' = A + B \cdot (\log P)/Ps + C \cdot (\log P)/Ps^2 \qquad \text{.....(Eq.4.9)}$$

$$\ln k' = A + B \cdot (\log P)/Ps + C \cdot (\log P)^2/Ps^2 \qquad \text{.....(Eq.4.10)}$$

4.3 EXPERIMENTAL

Chromatographic studies were performed using either a Spectra-Physics model SP8100 liquid chromatograph with model SP8440 UV-VIS detector and model SP4200 computing

integrator (Spectra-Physics, St. Albans, U.K.) or a modular system assembled from a ConstametricIII pump (Milton Roy, Stone, U.K.), Rheodyne 7125 injection valve, and a UV-LC detector (Pye-Unichem). Methanol, acetonitrile and tetrahydrofuran were HPLC grade (Fisons, U.K.), and HPLC grade water was obtained from Milli-Q water system (Millipore, Harrow, U.K.).

Data Set 3:

A 50x4.6mm i.d. column was packed with 3μm Hypersil-ODS (Shandon, Cheshire, U.K.) maintained at 40°C, flowrate 1 ml/min and UV detector at 254nm. For the methanol-water mixtures, five compositions were used(28,42,58,74 and 90 %v/v methanol) and the isoeluotropic equivalents[4.19] for acetonitrile and THF. Five non-ionic solutes were chosen for their range of logP values (given in parentheses), namely benzyl alcohol (1.10), benzonitrile (1.56), benzene (2.15), chlorobenzene (2.84) and benzophenone (3.58), and injected individually, in triplicate, dissolved in 25% methanol.

Data Set 4:

A 150x4.6mm i.d. column was packed with 6μm Zorbax-ODS (DuPont) maintained at 35°C, flowrate 1 ml/min and UV detection at 254nm. For the methanol-0.0025M phosphate buffer(pH 6.9) compositions, 50,60 and 70% v/v methanol were used with their equivalents for acetonitrile and THF. The five non-ionic solutes were

benzonitrile(1.56), benzene(2.15), toluene(2.718), naphthalene(3.2) and biphenyl(4.09), dissolved in 50 %v/v methanol, and injected individually in triplicate.

Data processing and analysis was performed using statistical software (MINITAB®) and Honeywell 68 DPS level 2 via RJE or GEC 63/40 computers.

4.4 RESULTS AND DISCUSSION

An examination of Table 4.2(a) shows that all equations showed significant "fit" to the experimental data as judged from the F-statistics, even for Eq.4.10 for which F-ratio was lowest (F=127) is significant ($F(k=2,n-k-1=42, \gamma= 99.9\%) < 8.09$)[4.22]. The minimum coefficient of determination (r^2) was 73.4% for Eq.4.10 with Data set 3.

Since experimental data may include some accidentally large errors a "trimming" step was carried out in order to remove such "outlier" observations. The criterion used for this purpose was the calculated studentised residual(SR) value for each observation. This is a procedure in which the estimate of standard deviation of each data point is obtained to calculate t-statistics for normality from the residual value(error). Generally, if t-value is greater than 2.0 then the observation (dependent variable) is considered as an "outlier".

Statistical analysis was again performed for the "trimmed" data, and the results appear in Table 4.2(b). It was found that there were few outliers, and removing them improved the values for coefficient of determination (r^2), standard deviation(s) and F-ratio, although, relative statistics for different equations did not alter considerably.

Out of six equations(Eq.4.5-4.10) only three, viz. Eq.4.7, 4.9 and 4.10 were able to obtain regression coefficient without showing multicolinearity(see Table 4.2(a) and 4.2(b) where '-' indicates multicolinearity). This finding implies that only these equations are statistically robust as compared to the rest of the equations.

In order to make a further choice from these equations (Eq.4.7, 4.9 and 4.10), the weighted (according to number of data points) F-ratio, higher coefficient of determination(r^2) and lower standard deviation (s) were considered as statistical criteria. It is apparent that Eq.4.7 and Eq.4.9 are outstanding in these terms. However, Eq.4.7 is able to explain (higher r value for Data Set 2) a large proportion of the pooled data. Eq.4.7 also shows higher coefficient of determination (r^2=96%) for Data set 2 (highest number of observations) as compared to Eq.4.9 (r^2=89%).

Further support for Eq.4.7 as a "good" general model comes from an examination of regression coefficients and the error associated with their estimation. The regression coefficients and t-ratios appear in

Table 4.3(a,b) and a summary of regression coefficients and t-statistics for Eq.4.7 and Eq.4.9 appear in Table 4.4. It is apparent that coefficients (A,B and C) for Eq.4.7 are consistent, i.e., they show similar magnitude and sign for all data sets in constrast to Eq.4.9. Such consistency of the estimated coefficients implies that a common "trend" of chromatographic process is occuring in different but related RPLC systems, governed by the hydrophobic parameters of the solutes and solvents.

Additionally, the t-ratios(ta, tb and tc) for each coefficient is higher for Eq.4.7 as compared to those for Eq.4.9. This indicates that the coefficients for Eq.4.7 could be estimated with less error so that a higher confidence limit could be assigned.

To summarise the points which favoured the selection of Eq.4.7.

1. It is a simple equation as compared with others considered in this study.

2. It explains upto 95-96% of variation(r^2) in lnk'.

3. It gives regression coefficients which are similar in their magnitude and sign for data obtained under different chromatographic systems. Therefore making it easier for wider application.

4. All regression coefficients could be estimated with significantly less error as shown by higher t-statistic.

It was therefore decided to accept Eq.4.7 as a general model describing the retention of the solutes in RPLC on the basis of the physico-chemical parameters of the solute and solvent. One question that may be raised is "Does this model(Eq.4.7) provide relationships that are generally accepted for RPLC, e.g. Eq.4.1 and Eq.4.2. This can be shown as follows:

For a given solute (logP=X) Eq.4.7 reduces to Eq.4.11.

$$\ln k' = A + B \cdot X \cdot 1/Ps + C \cdot 1/Ps^2 \qquad \ldots(Eq.4.11)$$

But X=constant and if B'=B·X, then

$$\ln k' = A + B' \cdot 1/Ps + C \cdot 1/Ps^2 \qquad \ldots(Eq.4.12)$$

Substituting 1/Ps by Ø in Eq.4.12 leads to Eq.4.2.

For a given mobile-phase (1/Ps=Y) Eq.4.7 reduces to Eq.4.13.

$$\ln k' = A + B \cdot Y \cdot \log P + C \cdot Y \qquad \ldots(Eq.4.13)$$

But Y=constant, and if

B'=B·Y,

C'=C·Y and

A'=A + C', then

$$\ln k' = A' + B' \cdot \log P \qquad \ldots(Eq.4.14)$$

Eq.4.14 is same as Eq.4.1. Thus it evident that Eq.4.7 is a general model which holds true for specific cases.

A graphical comparision of observed (experimental) retentions (lnk') and calculated retentions using Eq.4.7 for Data set 1 to 4 appear in Fig.4.1. It is evident that a good correlation between observed and predicted retention exists over a wide range of capacity factor values.

Having established that Eq.4.7 is a statistically sound representation of the dependence of retention (lnk') on the hydrophobic parameters of the solute (logP) and solvent (Ps) for different data sets, it is appropriate to interpret the physical significance of Eq.4.7.

The quadratic dependence of lnk' on 1/Ps in Eq.4.7 reflects the relationship given by Eq.4.2 which was derived theoretically[4.18] for the solvent volume fraction (Ø) in the mobile-phase and it was shown in this study that Ø and 1/Ps are linearly related(Eq.4.3). It should be remembered that such a quadratic relationship is only required for a wide range of k'(k'>10) otherwise a linear relationship is adequate (see Eq.3.1). The direct dependence of lnk' on logP shows the most commonly observed fact that retention is dependent on the hydrophobic parameter(logP) of the solute. The significance of the ratio logP/Ps can be explained as follows.

For a given stationary phase the hydrophobic property (length of hydrocarbonaceous chain) and the probability(% carbon loading) of interaction are predefined, i.e. a scale of hydrophobic retention is set for neat water(buffer) as a solvent system. This

scale will be reduced for a less polar solvent system. In other words a solute will show different retention under different solvent systems. However, if the ratio of retention to the solvent polarity is calculated then the ratio would not change. This ratio reflects the hydrophobic parameter (logP) of the solute.

When considering the logP/Ps ratio, it is apparent that the greater the logP value of the solute the greater is the solvent partition coefficient (Ps) required to provide the same retention (lnk'). Thus it seems that taking such a ratio provides an unique relative retention scale for the given stationary phase. As mentioned earlier the quadratic relationship of solvent property with lnk' is only useful when k' is greater than 10 or sometimes 20. Therefore Eq.4.7 can be reduced to Eq.4.15 for k' less than 20.

$$\ln k' = A + B\cdot(\log P)/Ps \qquad \ldots(\text{Eq.4.15})$$

In order to understand this relationship further a comparison of conventional plots of lnk' vs. logP and a plot of lnk' vs. (logP)/Ps is shown in Fig.4.2(a) and 4.2(b) respectively. Fig.4.2(a) shows that retention of solutes in different solvent conditions gives different linear plots (curves 1,2 and 3 for increasing 1/Ps or decreasing Ø values). That is, different retention scales are required for different solvent-systems. However if a graph of lnk' vs. (logP)/Ps (Eq.4.15) is made, this accomodates differences between solvent-systems and provides only a single retention scale as shown in Fig.4.2(b). In

other words the coefficients for Eq.4.7 or Eq.4.15 uniquely reflect the chromatographic system's characteristics (stationary-phase) independent of the solvent systems (mobile-phase) employed. Therefore it is probably for this reason that the estimated coefficients of Eq.4.7 for different data sets are similar as shown in Table 4.4.

Eq.4.7 is therefore in complete agreement with the well-known and observed relationship between solute retention and content of the organic modifier in the mobile phase, and provides an explanation of this relationship.

As mentioned earlier it was the aim of this part of the study to examine if chromatographic(RPLC) behavour of unionised solutes in any solvent-system can be predicted employing the hydrophobic parameter of the analyte and the solvents used. It has been demonstrated that Eq.4.7 could be used as a general model for this purpose. Since this relationship is solely dependent on the hydrophobic parameter it is implied that the predictions may provide approximate if not very accurate , because other factors also influence the retention mechanism. However such a model(Eq.4.7) could become useful as a general method development guide. A suggested procedure is as follows.

For a given chromatographic system (stationary-phase) the coefficients for Eq.4.7 are estimated by the analysis of a few solutes sufficiently different in their logP values, under different solvent strengths (employ 1/Ps scale) for different solvents, i.e. MeOH, ACN, THF etc. Once the coefficients (A,B and C) are known they can be used to predict lnk' values for other analytes whose logP value is known. The experimental logP values of the compounds are easily available[4.23] or can be determined experimentally or can be calculated theoretically. The logPs or 1/Ps values can be calculated using the programme "STRENGTH" (Appendix A). Fig.4.3 shows the contour-type plots of predicted lnk' for Data set 1 to 4, where "*" indicates the region of $1<k'<10$. Fig.4.4 shows an average of all the four Data sets. Once such plots are made for a type of stationary-phase it would become very easy to find "initial" solvent conditions such that the retention would fall in the k' range of either 1 to 10 or 1 to 20. Consider a hypothetical sample containing few known solutes whose logP values range from 2 to 4.5. We wish to determine a solvent system able to elute all components of this mixture between $k'=1$ to 10 or 1 to 20. Suppose we wish to use the stationary-phase employed in Data set 1 for which we have obtained coefficients for Eq.4.7. The arrangement for deciding appropriate mobile phase is presented in Fig.4.5. There are three contour lines drawn. These corresponds to $k'=1$, 10 and 20 respectively from left to right (decreasing solvent strength). Obtaining a

projection of point logP=2 (Y-axis) from contour line k'=1 on X-axis(1/Ps=11.13) as indicated by 'j'. Similar projection for logP=4.5 point is taken from k'=10 on X-axis (1/Ps=10.61), as indicated by 'i'. It is apparent that solvent required for eluting solute with logP=4.5 is "stronger" than the solvent required for eluting solute with logP=2 at k'=1. In other words, if solvent with 1/Ps=10.61 is employed than the first peak will elute with k'<1. It is therefore evident that if we wish to elute all compounds in 1<k'10 range it would not be possible. Neverthless, all solutes may be eluted in 1<k'<20 range as indicated by the projection of point logP=4.5 from k'=20 contour line on X-axis (1/Ps=12.33). This is graphical approach, but a numerical method could be used.

4.5 CONCLUSION

A stochastic model of retention behaviour of solutes solely based on the hydrophobic parameter of the solute(logP) and the solvent system(Ps) has been derived using experimental data. The model was found to be statistically sound. The empirical regression coefficients for this model were consistent and they could be estimated with less error for all data sets analysed.

The ratio of logP and Ps has been found to be an important factor. A distinct advantage offered by the present approach is that it allows all retention data

for different solutes analysed under different solvent systems, for a given column, to be combined in a single model. It is hoped that the model presented in this study would be useful to analysts in method development because the logP values are readily available or can be theoretically calculated.

TABLE 4.1

STATISTICS FOR THE RELATIONSHIP BETWEEN Ø AND 1/Ps (Eq.4.3)

Solvent	Ø	A	B	n	r	s	F
MeOH	0 to 1	1.04	-0.0569	101	0.998	0.015	42816
ACN	0 to 1	1.07	-0.0439	101	0.999	0.005	51069
THF	0 to 1	0.92	-0.0378	101	0.999	0.006	37714

Where,

n; number of observations,
r; correlation coefficient
s; standard deviation, and
F; F-ratio.

TABLE 4.2(a)

STATISTICAL ANALYSIS FOR EQ.4.5-4.10
FOR FOUR DATA SETS.

		All Observations			
Eq.	Data	n	r	s	F
4.5	1	-	-	-	-
4.5	2	1176	0.951	0.7516	3455
4.5	3	145	0.947	0.4025	409
4.5	4	45	0.961	0.2572	167
4.6	1	-	-	-	-
4.6	2	1176	0.971	0.5842	5401
4.6	3	145	0.977	0.2668	755
4.6	4	45	0.973	0.2203	174
4.7	1	131	0.973	0.2346	1113
4.7	2	1176	0.967	0.6110	8045
4.7	3	145	0.969	0.3111	1040
4.7	4	45	0.971	0.2231	337
4.8	1	-	-	-	-
4.8	2	1176	0.971	0.5847	7200
4.8	3	145	0.972	0.2943	803
4.8	4	45	0.971	0.2258	220
4.9	1	131	0.972	0.2375	1112
4.9	2	1176	0.913	0.9866	2869
4.9	3	145	0.974	0.2861	1313
4.9	4	45	0.972	0.2171	360
4.10	1	131	0.967	0.2572	934
4.10	2	1176	0.954	0.7227	6268
4.10	3	145	0.857	0.6444	196
4.10	4	45	0.926	0.4382	127

Where,

-; multicolinearity observed,
n; number of observations,
r; correlation coefficient
s; standard deviation, and
F; F-ratio.

TABLE 4.2(b)

STATISTICAL ANALYSIS FOR EQ.4.5-4.10
FOR FOUR DATA SETS

Eq.	Data	Outliers Removed* n	r	s	F
4.5	1	-	-	-	-
4.5	2	1112	0.967	0.5385	5199
4.5	3	135	0.956	0.3239	464
4.5	4	42	0.974	0.2040	232
4.6	1	-	-	-	-
4.6	2	1112	0.983	0.4022	6587
4.6	3	135	0.985	0.2187	1060
4.6	4	42	0.983	0.1738	269
4.7	1	127	0.976	0.2218	1232
4.7	2	1112	0.981	0.4278	13013
4.7	3	135	0.973	0.2667	1211
4.7	4	42	0.979	0.1806	443
4.8	1	-	-	-	-
4.8	2	1112	0.983	0.4072	8824
4.8	3	135	0.979	0.2477	1024
4.8	4	42	0.979	0.1824	293
4.9	1	127	0.974	0.2313	1147
4.9	2	1112	0.945	0.7382	5043
4.9	3	135	0.984	0.2262	2081
4.9	4	42	0.987	0.1505	694
4.10	1	127	0.974	0.2292	1119
4.10	2	1112	0.967	0.5332	8368
4.10	3	135	0.893	0.5695	265
4.10	4	42	0.948	0.2985	179

* Note that n is decreased

Where,

-; multicolinearity observed,
n; number of observations,
r; correlation coefficient
s; standard deviation, and
F; F-ratio.

TABLE 4.3(a)

REGRESSION COEFFICIENTS AND t-RATIOS FOR Eq. 4.5-4.10 FOR ALL OBSERVATIONS

Data 1

Eqn.	A	ta	B	tb	C	tc	D	td	E	te
4.5	--	--	--	--	--	--	--	--	--	--
4.6	--	--	--	--	--	--	--	--	--	--
4.7	-2.1108	-17.25	0.08556	44.51	0.0025	5.84	--	--	--	--
4.8	--	--	--	--	--	--	--	--	--	--
4.9	-1.4124	-21.96	0.04381	5.26	0.0025	5.48	--	--	--	--
4.10	-1.8461	-12.22	0.11352	11.44	-0.0004	-2.61	--	--	--	--

Data 2

Eqn.	A	ta	B	tb	C	tc	D	td	E	te
4.5	-3.2017	-34.83	0.9196	38.37	0.0467	3.54	0.0101	19.97	--	--
4.6	-0.9879	-9.22	-0.0610	-1.72	0.0747	27.73	-0.1206	-10.12	0.0101	25.6
4.7	-1.6610	-52.67	0.0672	53.35	0.0062	43.42	--	--	--	--
4.8	-1.1427	-19.68	0.0706	56.54	-0.1113	-10.45	0.0100	25.67	--	--
4.9	-1.4514	-27.80	0.1069	74.94	-0.0039	-0.35	--	--	--	--
4.10	-2.2421	-50.52	0.1928	66.90	-0.0226	-31.83	--	--	--	--

Data 3

Eqn.	A	ta	B	tb	C	tc	D	td	E	te
4.5	-1.1385	-3.41	0.5608	14.87	-0.0380	-0.68	0.0116	5.29	--	--
4.6	1.2657	4.45	-0.4421	-5.26	0.0826	13.45	-0.2570	-6.33	0.0133	9.1
4.7	-0.7449	-12.51	0.0479	21.55	0.0062	21.68	--	--	--	--
4.8	0.2403	1.00	0.4985	23.15	-0.1759	-4.20	0.0128	8.00	--	--
4.9	0.3608	5.90	-0.0347	-7.16	0.0059	24.12	--	--	--	--
4.10	-0.2212	-0.93	0.0618	3.73	0.0002	0.82	--	--	--	--

Data 4

Eqn.	A	ta	B	tb	C	tc	D	td	E	te
4.5	-3.374	-0.70	0.7832	18.10	0.1314	0.19	0.0071	0.30	--	--
4.6	0.531	0.12	-0.6185	-1.75	0.0981	3.98	-0.1419	-0.24	0.0071	0.35
4.7	-1.351	-7.91	0.0553	21.16	0.0063	7.70	--	--	--	--
4.8	-1.192	-0.28	0.0553	20.91	-0.0226	-0.04	0.0071	0.34	--	--
4.9	-0.027	-0.25	-0.0099	-1.08	0.0045	8.14	--	--	--	--
4.10	-0.195	-0.40	0.0561	2.22	0.0001	0.22	--	--	--	--

Where, A,B,C,D and E are regression coefficients,
ta,tb,tc,td and te are t-ratio for each coefficient respectively, and
-- indicates either multicolinearity or absence of the constant

TABLE 4.3(b)

REGRESSION COEFFICIENTS AND t-RATIOS FOR Eq. 4.5-4.10 AFTER REMOVING OUTLIERS

Data 1

Eqn.	A	ta	B	tb	C	tc	D	td	E	te
4.5	--	--	--	--	--	--	--	--	--	--
4.6	--	--	--	--	--	--	--	--	--	--
4.7	-2.0685	-17.77	0.0850	46.49	0.0024	5.84	--	--	--	--
4.8	--	--	--	--	--	--	--	--	--	--
4.9	-1.4205	-22.66	0.0459	5.65	0.0024	5.32	--	--	--	--
4.10	-1.8274	-13.49	0.1127	12.63	-0.0003	-2.83	--	--	--	--

Data 2

Eqn.	A	ta	B	tb	C	tc	D	td	E	te
4.5	-2.9921	-44.54	0.8619	47.04	0.0555	5.56	0.0089	23.19	--	--
4.6	-0.9984	-13.28	-0.9270	-3.29	0.0791	40.01	-0.1098	-12.82	0.0090	32.1
4.7	-1.5866	-70.34	0.0668	71.71	0.0057	55.11	--	--	--	--
4.8	-1.2072	-29.66	0.0727	77.24	-0.0950	-12.44	0.0089	31.71	--	--
4.9	-1.4974	-37.67	0.1060	93.93	-0.0009	-0.11	--	--	--	--
4.10	-2.1020	-63.43	0.1824	82.71	-0.0214	-38.80	--	--	--	--

Data 3

Eqn.	A	ta	B	tb	C	tc	D	td	E	te
4.5	-0.9145	-3.32	0.4657	14.48	-0.0327	-0.71	0.0109	5.94	--	--
4.6	1.2501	5.33	-0.4603	-7.04	0.0858	16.53	-0.2554	-7.63	0.0131	10.6
4.7	-0.7013	-13.13	0.0470	22.80	0.0061	24.51	--	--	--	--
4.8	0.1848	0.90	0.0491	25.60	-0.1631	-4.57	0.0124	9.00	--	--
4.9	0.2180	4.28	-0.0244	-6.06	0.0056	27.62	--	--	--	--
4.10	-0.1776	-0.84	0.0513	3.45	0.0004	1.92	--	--	--	--

Data 4

Eqn.	A	ta	B	tb	C	tc	D	td	E	te
4.5	-5.076	-1.28	0.8079	21.78	0.3899	0.69	-0.0028	-0.14	--	--
4.6	0.278	0.08	-0.5780	-1.92	0.0996	4.75	-0.1217	-0.25	0.0063	0.3
4.7	-1.222	-8.52	0.0567	24.81	0.0055	8.06	--	--	--	--
4.8	-2.897	-0.82	0.0567	24.56	0.2371	0.47	-0.0028	-0.16	--	--
4.9	-0.185	-2.39	-0.0052	-0.77	0.0045	10.87	--	--	--	--
4.10	-0.501	-1.18	0.0730	3.31	-0.0001	-0.45	--	--	--	--

Where, A,B,C,D and E are regression coefficients,
ta,tb,tc,td and te are t-ratio for each coefficient respectively, and
-- indicates either multicolinearity or absence of the constant

TABLE 4.4

SUMMARY OF REGRESSION COEFFICIENTS
FOR Eq.4.7 and Eq.4.9

Eq.4.7

Data	A	B	C	ta	tb	tc
1	-2.0685	0.08499	0.00237	-17.8	46.5	05.8
2	-1.5866	0.06675	0.00568	-70.3	71.7	55.1
3	-0.7013	0.04699	0.00608	-13.1	22.8	24.5
4	-1.2222	0.05669	0.00551	-08.5	24.8	08.1
Ave.	-1.3947	0.06386	0.00491	-	-	-

Eq.4.9

Data	A	B	C	ta	tb	tc
1	-1.4205	0.04590	0.00240	-22.7	05.7	05.3
2	-1.4974	0.10600	-0.00090	-37.7	93.3	-00.1
3	0.2180	-0.02440	0.00560	04.3	-06.1	27.6
4	-0.1850	-0.00520	0.00045	-02.4	-00.8	10.9

Note: A,B,C are regression coefficients and
ta, tb and tc are t-ration respectively.

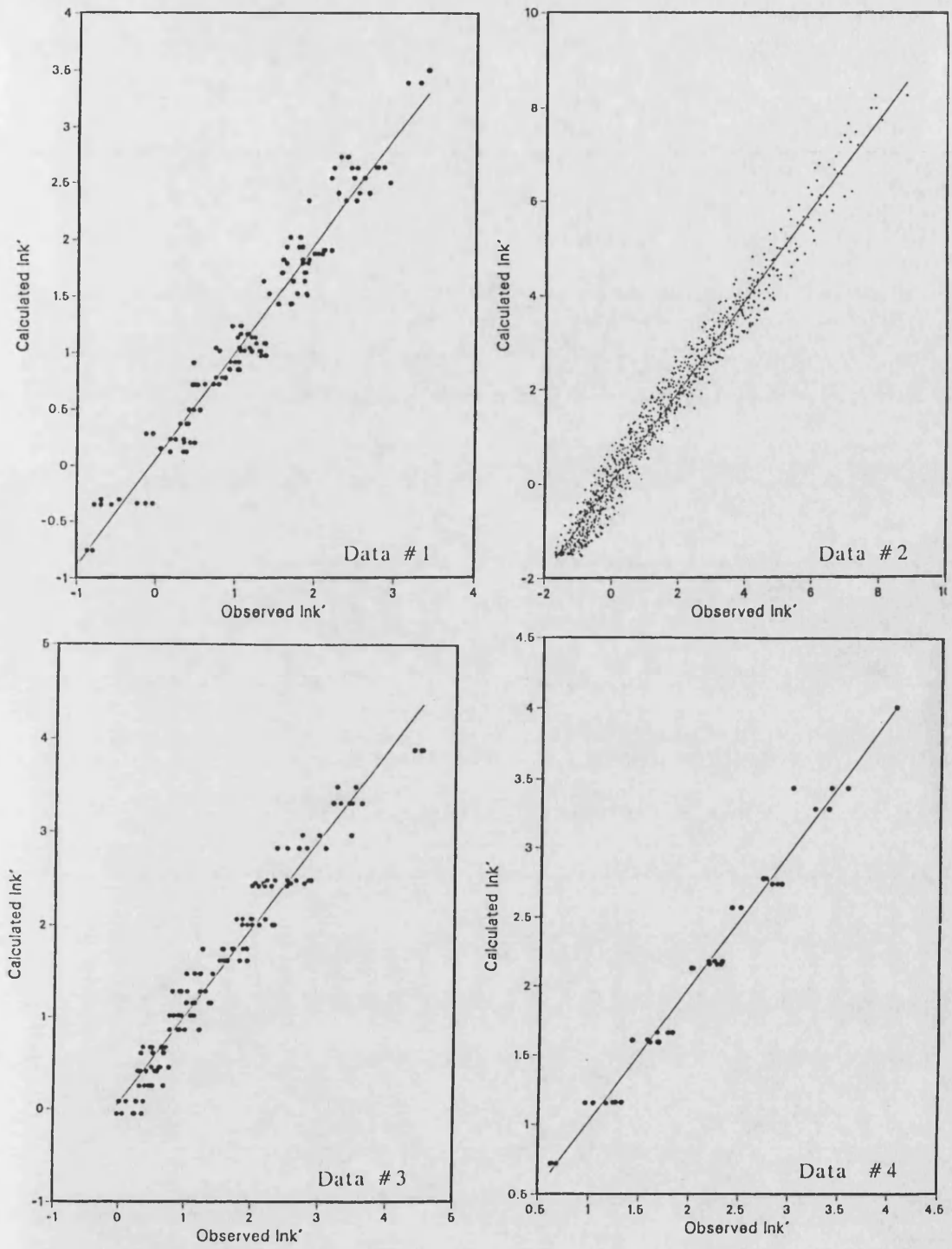
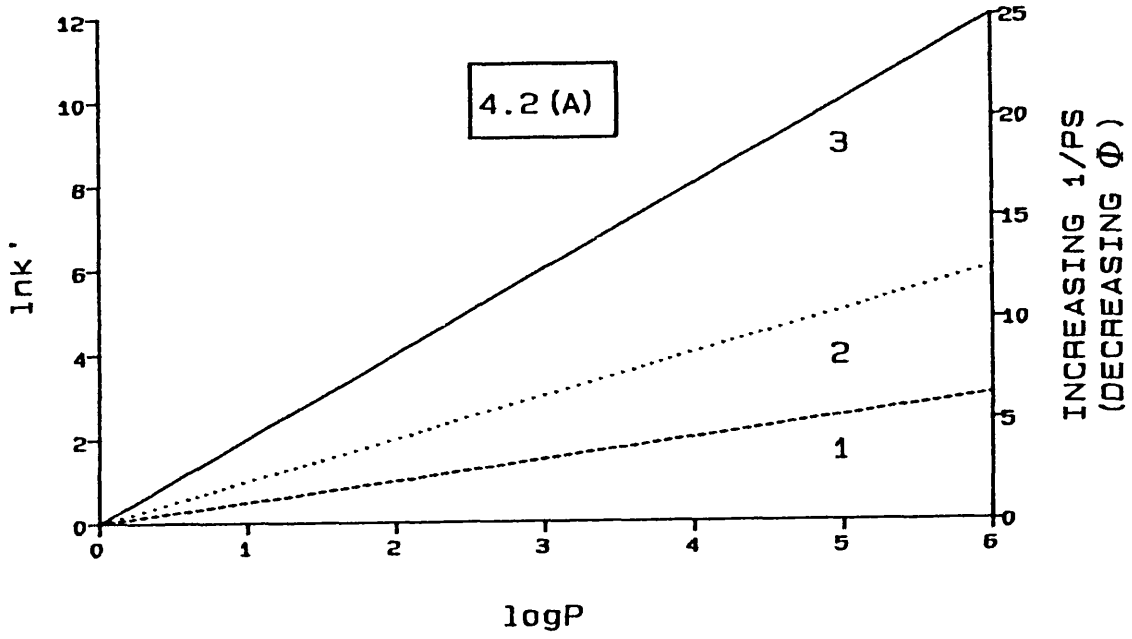


Fig.4.1 Plots of experimental (observed) and calculated retentions (lnk') using Eq.4.7 for four data sets.



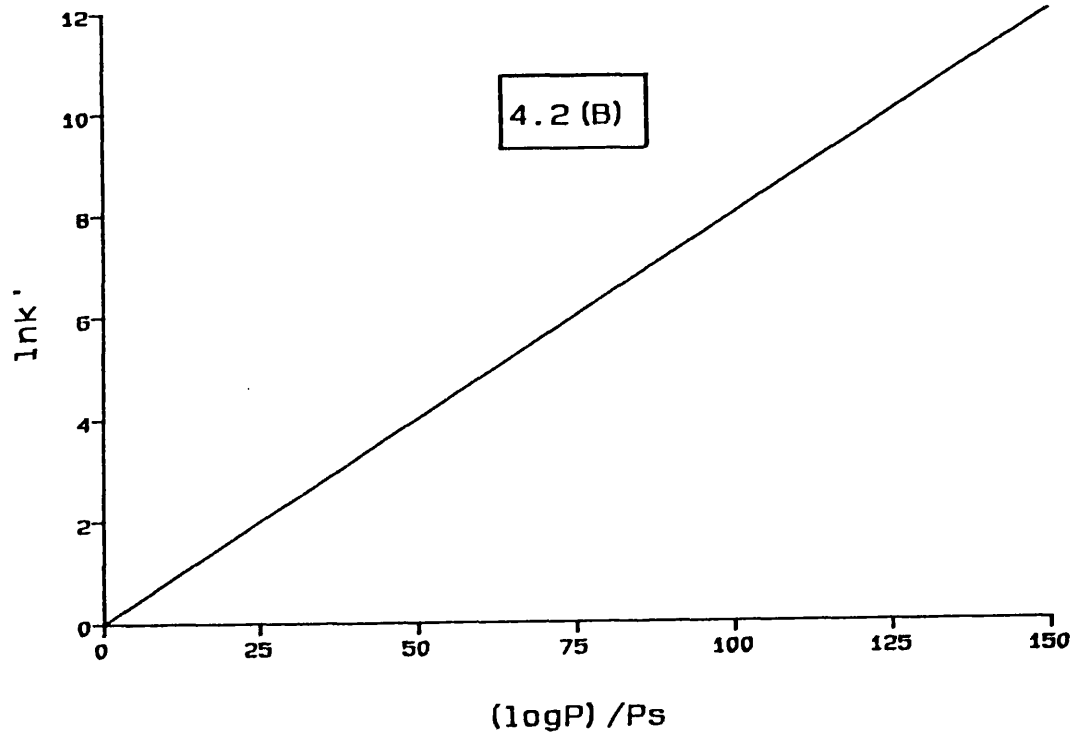
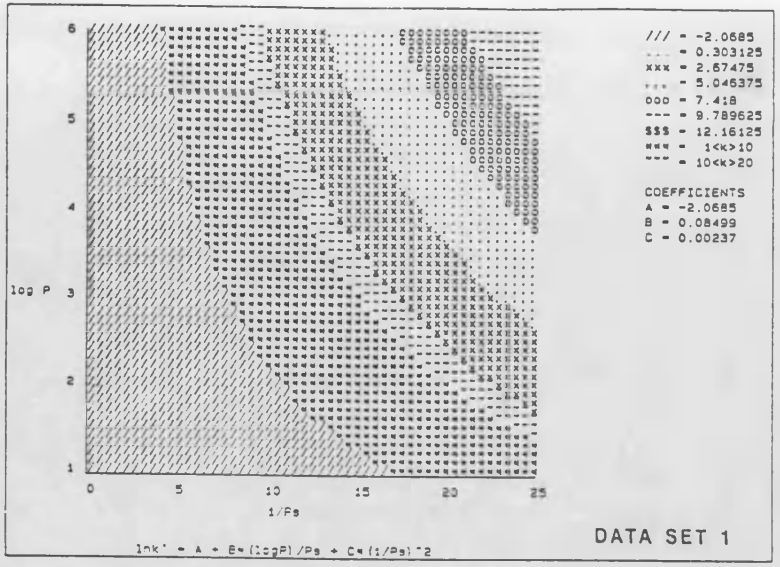
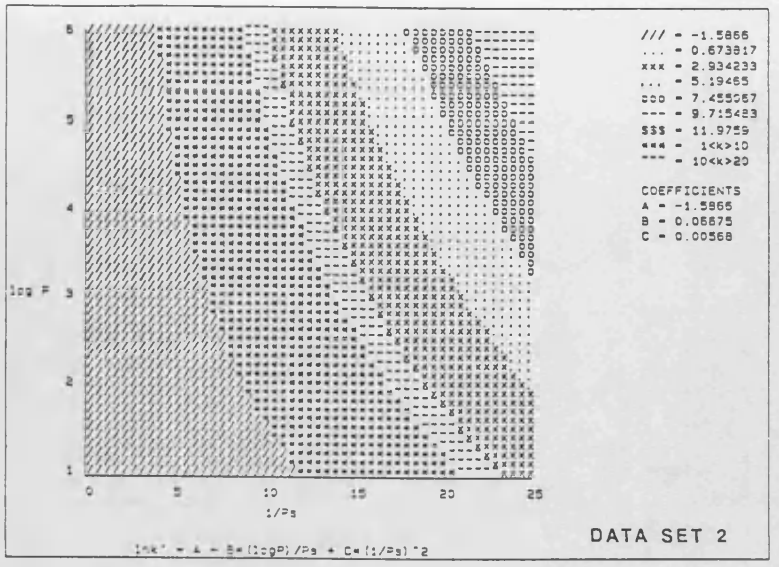
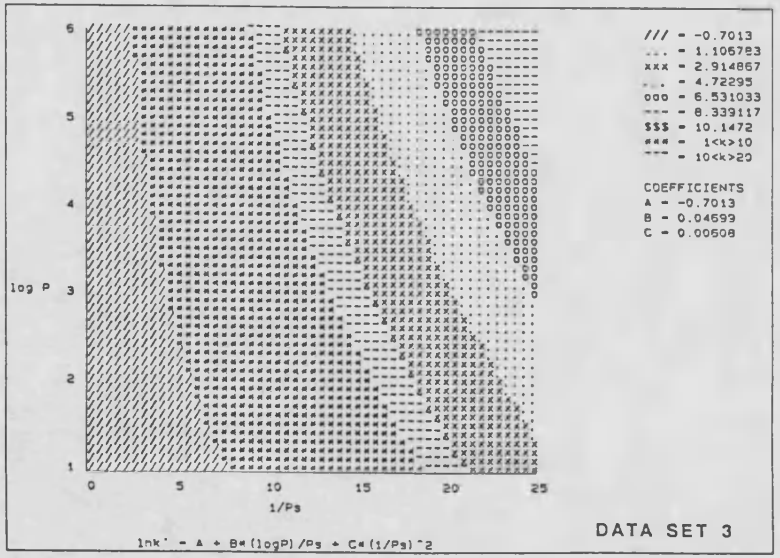


Fig.4.2 Comparison of conventional and proposed retention scale. (A); Conventional, abd (B) scale suggested in this study. Note that for conventional plots there are different curves (shown as 1,2 and 3) for different solvents for the relationship between the hydrophobic parameter and retention in contrast to the proposed scale.







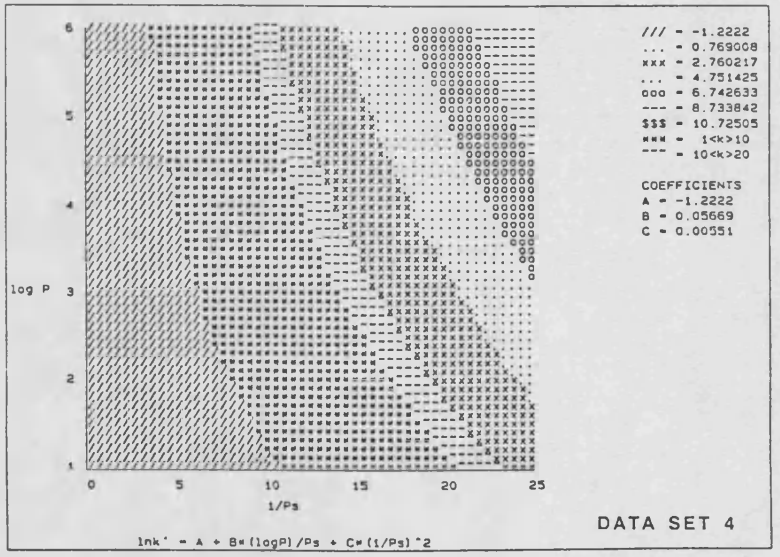


Fig.4.3 Contour-type maps, based on Eq.4.7, showing regions of ideal retentions (1<k'<10 and 10<k'20) for Data sets 1 to 4.

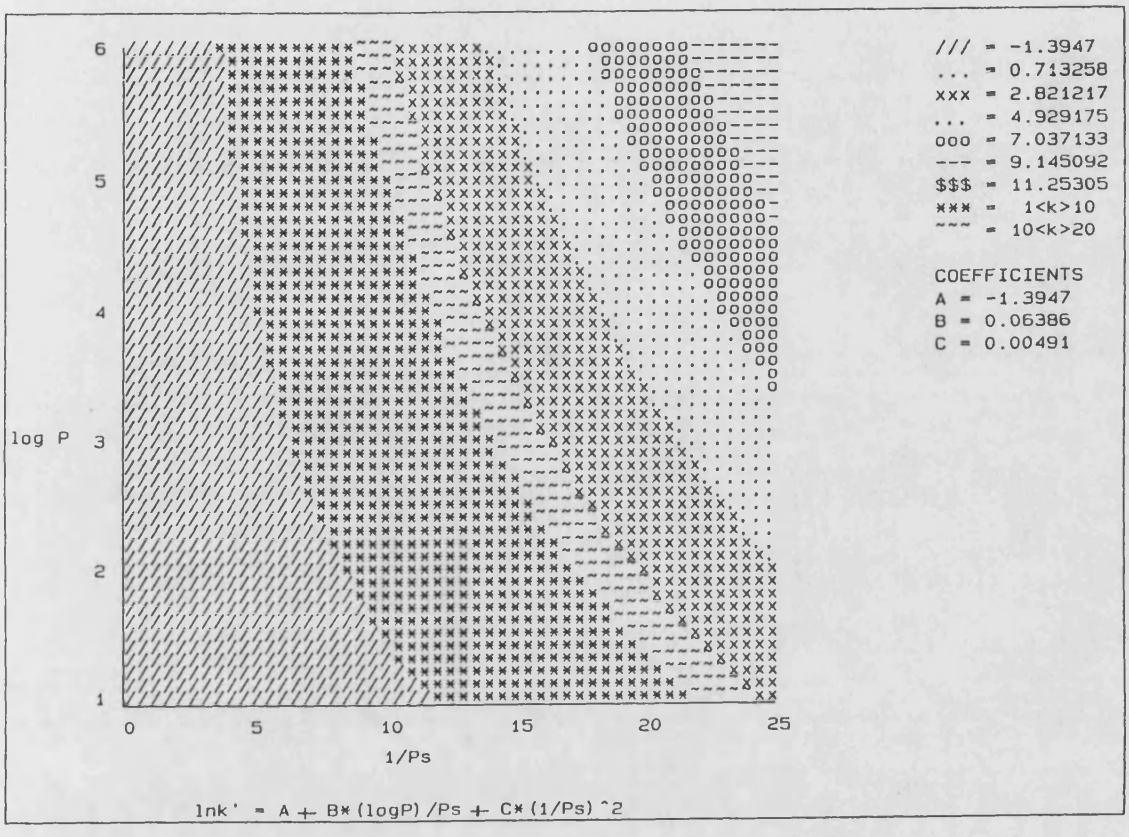


Fig.4.4 Contour-type map , based on Eq.4.7, showing regions of ideal retentions (1<k'<10 and 10<k'20) for average of Data sets 1 to 4.

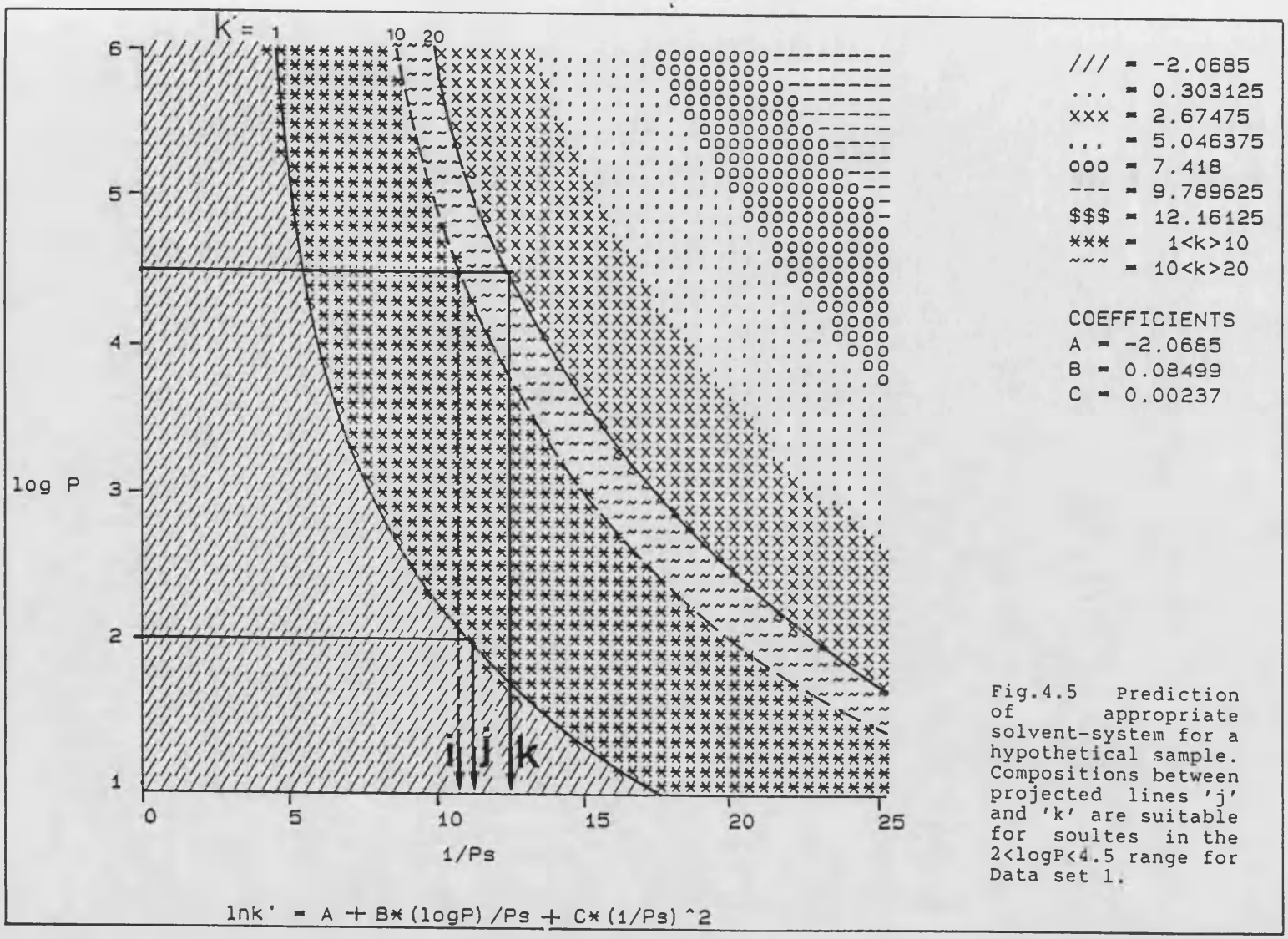


Fig.4.5 Prediction of appropriate solvent-system for a hypothetical sample. Compositions between projected lines 'j' and 'k' are suitable for soultes in the 2<logP<4.5 range for Data set 1.

CHAPTER 5

OPTIMISATION OF CHROMATOGRAPHIC SELECTIVITY IN THE ISO-ELUOTROPIC SOLVENT PARAMETER SPACE

5.1 INTRODUCTION

There can be no doubt that reversed-phase high performance liquid chromatography has become one of the most powerful and widely used tools available to the analytical chemist. It has been used for qualitataive and quantitative analysis of simple to moderately complex mixtures. The actual analysis is then dependent on the physico-chemical properties of the components (solutes, solvents and stationary phase), the complexity of the sample matrix and the experimental conditions chosen by the analyst.

The factors that influence the analysis are the nature of the stationary phase, the qualitative and quantitative composition of the mobile phase, the mode of separation i.e. isocratic or gradient elution, flow rate, temperature, pH, buffer concentration and in the case of ion-pair chromatography, the concentration of ion-pair reagent. Considering the many variables concerned it is not surprising that the development of an HPLC method is a challenge to chromatographers. In fact it is these variety of factors which gives RPLC

its power of analysis, as separation(s) could be achieved by tailoring one, few or all variables.

Normally many of the above mentioned factors are preselected and maintained at a constant level whilst changing only those factors which potentially offers maximum variation in the selectivity. A direct application of this concept is the change in the mobile phase composition because it offer significant changes in the selectivity. A measure of selectivity is resolution, which can be expressed as follows:

$$Rs = 1/4 \cdot (\alpha - 1) \cdot N^{1/2} \cdot k'/(k'+1) \qquad .....(Eq.5.1)$$

where,

Rs = resolution factor,

α = selectivity factor,

N = plate number and

k' = capacity factor.

In the above equation, N(i.e. column packing and length) is noramlly preselected. However the value of Rs can be influenced by altering the strength (quantitative composition) of the mobile phase which controls the capacity factor. More importantly a change in selectivity (α) is offered by altering the qualitative composition of the mobile phase. Solvent strength/selectivity changes offers a number of advantages in resolution of the solutes, because

1. it is one of the most influencial variables,

2. it is easy to vary,

3. it is possible to control it within experimental error,

4. it frequently influences the separation in an unpredictable and non-monotonic way (unlike some other variables) and therefore cannot be preselected.

Therefore once other parameters are selected, from previous experience or other systematic approaches, the aim of the analyst is to obtain optimum analysis by altering the qualitative and quantitative composition of the mobile phase, i.e. solvent optimization. A general definition of the optimum and optimization is given below.

OPTIMUM:
It is a value or set of values of the dependent variable(s) which satisfies a set of criteria under the given/chosen boundaries of a selected set of independent variables.

OPTIMIZATION:
It is an iterative/non-iterative search process by which the OPTIMUM is located and its coordinates(i.e. the values of independent variables) are established.

Thus an optimum, if any, is enclosed in the parameter space selected by the analyst. If an optimum is not found in this space then either a redefinition of the parameter space is inevitable or the constraint parameters for accepting the optimum should be relaxed. However, a practical definition would be to achieve the best possible results under the given/chosen parameter space.

The problem of finding the appropriate composition (qualitative and quantitative) of the solvent, within solvent parameter space corresponding to an optimum/optima is at the heart of chromatographic selectivity optimisation.

Traditionally the optimisation of binary solvent systems has been achieved by "trial-and-error" methods. This is probably an efficient way of method development if the sample mixture consist of a few chemically distinct compounds, using binary solvents. However the difficulty of the analysis increases steeply for complex mixtures and where either ternary or quaternary solvent systems are required or employed. In such cases the traditional approach either cannot be used or is too inefficient to use practically.

One of the earliest attempts to use a graphical technique for optimisation was by Laub and Purnell[5.1]. They used the "window diagram" technique for optimising stationary phases in GLC, which is in some way similar to the solvent optimisation in RPLC.

The advent of computers, especially the microcomputer and microprocessor controlled liquid chromatographs, which allowed automated but controlled analysis, made it possible to use some of the established mathematical, statistical or numerical techniques to attack the optimisation problem in HPLC. It offered not only the possibility of efficient method development but also provided a means of simulating HPLC analysis or in other words producing "synthetic chromatograms" from the empirical rules or models learned from a small but sufficient set of experimental data. A number of either off-line or on-line(integral) computer-aided solvent optimisation methods are now available. A list of such methods is given in Table 5.1.

5.2 THEORETICAL

5.2.1 Introduction

An optimisation method, also known as a chemometric technique, may be based on either deterministic or empirical principles. Attempts towards the development of a deterministic model are limited in number and also in their success rate. Few of the successful reports are available in literature[5.29-5.31]. This approach cannot be used when the nature of the constituents of a sample mixture is not known.

On the other hand, non-deterministic approaches (listed in Table 5.1) are useful in practice because a limited amount of information regarding the sample mixture is required and the model is developed , if any, or the search is done solely on the basis of the information obtained from the experimental data. Unfortunately, such models/algorithms are only useful for the specific conditions of solutes, stationary phase and solvent system for which they are developed. However a limited extension to similar conditions may be possible.

Before deciding on which experimental design or strategy to choose for an efficient solvent optimisation it is necessary to consider the question: "Which quantitative parameter should be chosen for optimisation ?"

5.2.2 Optimisation Criterion

Usually such a criterion is based upon selectivity (α or Rs) or a separation criterion (e.g. P[5.32] for adjacent peaks and an aggregrate single value parameter (the Response Function (RF)) is obtained to represent the overall 'quality' or 'goodness' of the chromatogram. The solvent composition is then optimised to achieve the best possible value of the response function.

A number of response functions have been reviewed[5.33-5.35], although none have been universally accepted as a general criterion. One disadvantage of using these parameters is that the

information about individual peaks is lost. A further complication might arise when the analysis time and total number of peaks are also included in such a parameter, which could even mislead the optimisation search.

The preceding discussion suggests that any such RF needs to be derived or used in such a way that it would not conflict with the chromatographer's judgement about the quality of the analysis and the efficiency for the separations of interest. However, as indicated by d'Agostino et al.[5.35], there is an inclination towards the use of resolution(Rs) based RF parameters such as COF, COC and RRP. Separation based parameters such as CRF are of limited value for multi-component separation in HPLC.

A comparative study by Debets et al.[5.36] showed that all the response parameters produce similar results, and pointed out two serious drawbacks of such functions. Firstly, the response changes very sharply when the number of detected peaks changes and secondly, without prior information on the total number of peaks expected most criteria did not give an optimal response when all peaks were base-line-resolved ($Rs > 1.8$). However the most serious problem occurs when the retentions of peaks cross-over. None of the response parameters are able to accomodate this effect which can only be overcome by using additional information, e.g. the use of diode-array (multichannel) UV detection as extensively studied by Fell et al.[5.37] or by using a

different detection principle as demonstrated by Gant and Perrone[3.38], in order to identify each peak i.e. peak-tracking. If peak identity could be established then optimisation could be achieved using the overlapping resolution map (ORM) technique, originally proposed by Scheffe[5.39] subsequently modified by Glajch et al.[5.15] or the "window-diagram" technique used by Laub and Purnell[5.1].

In the present study the Chromatographic Optimization Function (COF) has been selected as a response function.

5.2.3 Optimisation Methods

As stated earlier most of the optimisation methods are non-deterministic. A further division in this group is due to the mathematical principle used. In the heuristic approach, experiments are performed sequentially and the information gained is used to set new experiment(s) until such a time that an optimum is found. On the other hand, in the stochastic approach a fixed number of experiments are carried out and an empirical model is developed which is then used to predict the optimum.

The optimisation procedure may also employ either isocratic or gradient elution techniques. Again isocratic procedure can be iso-eluotropic or non-isoeluotropic depending upon whether optimization is sought in a single plane or a limited space enclosed by solvent vectors. In this study only the isocratic

iso-eluotropic approach has been examined i.e. an overall strength of the solvent is decided and a plane is defined which is iso-eluotropic (Fig.5.1) and an optimum is sought in this plane. It should not be confused with the global optimum in the whole tetrahedron space defined by four solvents.

A comprehensive classification of all the available optimization procedures is difficult. Fundamentally there are three parts of optimization procedure design. They are as follows:

1. Mathematical principle employed,

2. Chromatographic concept employed, and

3. Experimental technique used.

A schematic diagram presented in Fig.5.2 shows these components. It is apparent that most methods can be defined as a combination of any of the three components, e.g. sequential simplex method uses heuristic principle with isocratic experiments, whereas the Mixture Design Statistical (MDS) technique uses stochastic principle with iso-eluotropic mobile phases by iso-cratic elution.

A comparison of stochastic and heuristic approaches is presented in Table 5.2. It follows from this comparison that there are certain advantages associated with both approaches. It would, therefore, be useful to combine the concepts of both approaches into a single approach to provide a method having the merits

of both approaches, and more efficiently. Furthermore, such a procedure in association with the overlapping resolution map technique could offer very good methodology for the optimisation of chromatographic selectivity in HPLC.

In the present study a hybrid approach, i.e. employing the stochastic and heuristic principles, has been developed for isocratic iso-eluotropic solvent optimisation. It is also suggested that it also can be extended to non-isoeluotropic(isocratic or gradient) mobile phase optimisation techniques.

5.3 DESIGN OF OPTIMISATION STRATEGY

5.3.1 Iso-eluotropic Plane:

The initial conditions (solvent compositions) that define the solvent parameter space (iso-eluotropic plane) may be obtained as follows. A methanol-water mobile phase is found by "trial-and-error" that provide retention of all the solutes of the sample in the capacity factor range of 1 to 10 (or 1 to 20 if necessary).Methods proposed by Schoenmakers et al.[5.40] or by Haddad et al.[5.41] or if possible the method proposed in Chapter 4 may be used with advantage. This defines one of the vertices (A) of the iso-eluotropic plane shown in Fig.5.1. Vertices B and C corresponding to acetonitrile-water and tetrahydrofuran-water mixture are then predicted by the logPs parameter theory as proposed by Patel and Jefferies[5.42](Chapter 3). An adjustment in solvent

composition may be necessary at this stage so that the vertices correctly define the iso-eluotropic plane, i.e. the last retained peak has similar k' values for the mobile phases A,B and C.

5.3.2 Sampling Response Surface:

Once the iso-eluotropic plane is defined it is necessary to choose points in this plane from which to obtain the response, i.e. a chromatogram at each point whose COF parameter represents the response function value. There are three possible designs which can be employed depending upon which subsequent mathematical procedure is to be used for either interpolation or modelling. These designs are as follows and Fig.5.3(A),5.3(B) and 5.3(C) shows them graphically:

1. Fixed Point Design:- In this setup a fixed number of experiments are carried out and this number is dependent on the mathematical expression being used for the response surface modelling. An example of this approach is the Mixture Design Statistical (MDS) procedure, also known as simplex design. It was originally introduced by Claringbold[5.43] and Glajch et al.[5.15] were the first to use it for solvent optimization. A detailed discussion is presented by Snee[5.44]. It requires seven experiments, the data from which provide coefficients for the following expression (Eq.5.2).

$$COF = b1 \cdot A + b2 \cdot B + b3 \cdot C + b4 \cdot A \cdot B + b5 \cdot B \cdot C + b6 \cdot A \cdot C + b7 \cdot A \cdot B \cdot C \quad \ldots(Eq.5.2)$$

where, b1-b7 are coefficients and A-C are solvent compositions

This is a special cubic model recommended by Kurotori[5.45]. A simplex design is shown in Fig.5.3(A). This design is adequate for relatively simple or less "rugged" response surfaces. However a problem is likely to be encountered if the sample matrix is large leading to a topographically complex response surface. In such cases the simplex design would not be able to provide an accurate model. Therefore it would be ideal to have flexibility in the design such that it can be modified according to the experimental needs.

2. Open Design:- As the name implies, this is neither a fixed nor a flexible design. In fact there is no predefined strategy. The response surface is sampled according to the need, which may be an arbitrary one or dependent on the outcome of the previous experiment(s). Such a design is used in sequential simplex[5.46-5.48] and iterative regression[5.24] optimisation procedures.

3. Factorial/Grid Design:- In this design the response surface is sampled at fixed intervals (levels) of each factor (A,B and C) providing a grid structure of equally spaced points over the entire parameter space. It is similar to the simplex design in the sense that the number of experiments are fixed. However, unlike simplex the factorial design provides inherent flexibility so that the number of experiments can be varied

according to the anticipated complexity of the response surface. Furthermore, the data collected are amenable to interpolation as well as regression methods for modelling, which is another significant advantage.

Therefore the factorial/grid design has been chosen for the present work on the basis of the advantages outlined above.

The levels of A,B and C mixing are decided on the basis of the complexity of the sample matrix, however such a decision is purely empirical and intuitive; it could also be simply on the basis of the polynomial fit required to the response surface. Table 5.3 shows levels of mixing(n), number of experiments required(E) and the degree of polynomial(P) that would be fitted to the surface i.e. COF = f (B,C) with a degree of polynomial, P, with respect to each variable (note that A is a redundant variable).

In the present study a 5 level(n=5) mixing design is used requiring 15 analyses to be performed for optimisation. This design is shown in Fig.5.4. The values in each node indicate the chromatogram number, and the proportions of each of the solvent-water mixtures (pseudo-solvents A,B and C) are shown above the node.

5.3.3 The Response Function:

In order to evaluate the quality of the chromatogram it

is necessary to use a response function as discussed earlier . Such a function can be used as a guide to locate the optimum solvent composition(s). There are a variety of such functions (see section 5.5.2). In this work Chromatographic Optimisation Function (COF) was employed, although any other suitable function may be used. COF values for all chromatograms were calculated using Eq.5.3.

$$COF = \sum_{i=1}^{k} A \cdot \ln(Rs(i)/Rd(i)) + B \cdot (Tm - Tl) \qquad .....(Eq.5.3)$$

Where,

i = peak-pair number,

k = number of pairs,

A = weighting factor for each pair,

Rs(i)= resolution of ith pair,

Rd(i)= desired resolution of the ith pair,

B = weighting factor for time of analysis,

Tm = maximum acceptable analysis time, and

Tl = actual analysis time.

5.3.4 Response Function Evaluation:

The basic aim of the optimisation in the solvent parameter space is to establish those compositions which provide acceptable/satisfactory response function(COF) values. For this purpose a systematic study must be undertaken to examine the entire response surface delimited by the solvent-parameter vectors. But because only a limited amount of experimental information can be collected for the whole surface we have to use mathematical methods to obtain an estimate

of the true function value for those areas where experimental data are not available. In order to obtain such estimates either a model equation can be obtained by a regression method or linear interpolation can be used. It is known that regression methods provide an expression best describing the response plane and also provides estimates on possible experimental errors. If the largest source of the deviation of the true response plane from the mathematical model is experimental error, then the use of the regression may be beneficial. But if it is lack of fit between the model and the experimental data, then it may be detrimental. On the other hand, in absence of experimental error, the linear interpolatiion will give rise to errors in between the data points but provides an exact evaluation at those points where experimental data are available. As suggested by Schoenmakers[5.49] linear interpolation method should be prefered over regression methods if the experimental error in the data points is expected to be small relative to the error involved in the description of data with a regression model. Another major disadvantage of regression methods is that a relatively extensive series of polynomial regression must be examined so that coefficients that accurately describe the retentions as a function of solvent composition can be obtained, as expressed by d'Agostino et al.[5.35]. This is not only a disadvantage in itself but also poses another difficulty regarding the total automation of the optimization procedure. The

reason being that the automated regression-based self-modelling approach is not entirely reliable under all conditions and may provide relatively imprecise models. Such algorithms would put very high computing demands on the computer (especially microcomputers which are available as an integral part of LC systems). Furthermore, when such procedures are required to be used iteratively the whole optimisation procedure may slow down significantly as reported by Drouen et al.[5.26].

The preceding discussion clearly suggests that the interpolation technique has distinct advantages when compared to the regression technique. Therefore, in this study the interpolation technique has been adopted for the response function evaluation in the parameter space.

Conceptually, interpolation is a two-stage process. Firstly, it fits an interpolating function to the tabulated data points (spaced uniformly or otherwise). Secondly, it evaluates the function at the desired point by a series of approximations. Some of the interpolation methods that are described in the literature[5.50] are polynomial, rational function, bicubic and cubic spline interpolation. The former two methods do not require the information on the gradient values as compared to the later two. Rational function interpolation is especially useful for those functions which are only approximated by a rational function. However for simple and smoothly varying functions, such

as the response function in solvent optimization, polynomial interpolation technique is faster and adequate.

Polynomial interpolation has been used in this study with the assumptions that the response surface is continuous and smooth.

Interpolation methods are normally applicable to the univariate function. In the case of quaternary-solvent optimisation there are three pseudo-solvents, viz. MeOH-Water(A), ACN-Water(B) and THF-Water(C), due to the constraint that A+B+C=100, provides two variables, B and C. Thus optimisation in the solvent-parameter space is bivariate rather than univariate. Unfortunately there are no numerical methods available to interpolate directly in two-dimensions. However, univariate interpolation can be used in succession to obtain an interpolated function value for more than one dimension. This procedure requires a cartesian arrangement of the experimental data, as shown in Fig.5.5. But such an experimental design is impossible for the solvent-optimisation as there cannot be a solvent system containing 100% of both B and C. Such a difficuly may be overcome by the arrangement as shown in Fig.5.6. Here a reflection of the plane ABC is taken in the X-Y plane, i.e. all experimentally obtained COF values are filled in for those nodes for which the solvent compositions are imaginary (e.g. COF for 100%A (node1) is given to reflected node corresponding to 100%B + 100%C). This arrangement

provides a pseudo-factorial cartesian arrangement. Having arranged the data in the necessary format the interpolation in two-dimensions can be done as follows:

To determine a COF(B%,C%) value at a point P(X1,X2), as shown in Fig.5.5, the first step is to interpolate at each grid point parallel to the solvent B axis, i.e. estimates of COF(C%=constant(X2),B%) are obtained for different B% values. Now a second interpolation parallel to the C axis with B%=constant(X1) is performed providing desired value at P(X1,X2). Thus the COF(B%,C%) value can be obtained by bi-dimensional polynomial interpolation.

So in order to use a bi-dimensional interpolation polynomial technique we require a factorial design. Hence, for n combination of B and C there will be n(n+1)/2 experiments required and this arrangement is shown in Fig.5.4. The response is evaluated at each node(15 nodes for 5 level design).

5.3.5 Searching Optima:

Having decided upon a method of computing the response function at any desired solvent composition the next question is "How to locate the optima(global and local)?"

The simplest way is to do a grid search, as used by d'Agostino et al.[5.21], i.e. COF values are computed at sufficiently small intervals, say 4% of solvent, and the highest COF value is found. Then a second search

is done at 1% interval in that area only and a relatively more accurate estimate of the optimum is obtained. Interestingly, an up-hill simplex method can be employed to locate the optima with a very high degree of accuracy with a relatively minimum number of function evaluations. It was proposed by Nelder and Mead [5.51], also applied by Berridge[5.48] for experimental optimization in HPLC. The basic principle is shown in Fig.5.7 in which the simplex (a geometrical figure), here a triangle (abc) moves in the two-dimensional solvent parameter space such that it leads to the highest(local or global) COF value.

An analogy of this behaviour is that it is similar to the rolling of a ball on the side of a hill. If it encounters a pit in the path it stops(local optimum). However, if by chance it does not it will stop at the bottom of the hill(global optimum) as it seeks minimum(local or global) potential energy.

The advantages of using such a technique as an optimum locator are obvious as compared to grid search. Therefore, the up-hill simplex search was adopted for the optimisation strategy suggested in this work.

A up-hill simplex search can be performed starting from different solvent compositions. An internal routine then checks, by computing variance, whether an optimum is encountered in each search. If an optimum(either local or global) is not located within the maximum iteration limit then that search is terminated and a new search can be begun starting from a different

initial composition. This process is repeated as many times as the number of experiments(here 15) done to cover the whole triangular plane.

5.3.6 The Algorithm:

A complete description of the numerical algorithm for the optimisation would not be appropriate here. However a flow-diagram showing major processing and decision steps is shown in Fig.5.8. It would be necessary to mention that the simplex search routine was adopted from Nelder and Mead[5.51] with modifications in order to increase the efficiency of the process. The modification was introduced by making the step size dynamic, unlike the original routine where it was kept fixed throughout the search. The programme, called ORIENT, was written in FORTRAN77 as well as in PASCAL programming languages. ORIENT is an acronym for,

O ptimum
R esolution
I nvestigation using
E xperimental and
N umerical
T echniques

A listing of this programme in PASCAL language is provided in Appendix B.

5.4 EXPERIMENTAL

Chromatographic analysis was carried out using a Spectra-Physics Model SP8100 liquid chromatograph with a UV-VIS detector, Model SP8440, a computing

integrator, Model SP4220 and a stainless-steel column, 100x4.6 mm i.d., packed with 5μm Spherisorb-ODS2 (Shandon, UK). Methanol, acetonitrile and tetrahydrofuran were HPLC grade (Fisons, UK). Glass-distilled deionised water was used to prepare citrate-phosphate buffer (0.025M, pH 2.2). Buffer is refered to as water in this chapter. All injections were made by autoinjecter and the analyses were carried out at ambient temperature with mobile phase flow rate of 1ml/min and the effluent was monitored at 254nm.

A mixture of the compounds listed in Table 5.4 were dissolved in methanol-water mixture. This sample was then analysed in duplicate under the conditions required by the experimental design and also those predicted by the programme as optimum.

Data analysis was carried out on either Honeywell 68 DPS level 2 via RJE using FORTRAN 77 or on VAX 11/750 using PASCAL programmes.

5.5 RESULTS AND DISCUSSION

5.5.1 Optimisation With Composite Criterion(COF)

The first step for defining the iso-eluotropic plane was done by a "trial and error" method, i.e. the composition of methanol-water mixture providing retentions of all solutes in the capacity range of 1 to 10 as shown by Chromatogram 1 (now abbreviated Chrom/1) was established. This composition was 30% methanol in water. The actual k' values for Chrom/1 together with

other chromatograms appear in Table 5.5, where the k' value of the last retained peak (4-nitrobenzoic acid) is 10.62. Thus the vertex labelled 'A' in the multi-solvent space(Fig.5.1) was defined. The following compositions for ACN-water and THF-water were predicted by the logPs solvent strength parameter(cf. Chapter 3).

1. Chrom/1 : A= 30.00% MeOH-Water

2. Chrom/11 : B= 21.43% ACN -Water

3. Chrom/15 : C= 19.01% THF -Water

For vertex 'B'(Chrom/11) it is evident that predicted ACN-water composition is iso-eluotropic to MeOH-water (k' of last peak 9.02). However, in case of THF-water (vertex 'C') the retention of the last peak (k'=21.41) was significantly higher. The probable reason for such an unexpexted deviation is due to the acid character of all the solutes in the present study. It was shown by Bakalyar et al.[5.52] that for carboxylic-acid compounds a markedly different selectivity was shown in THF-water system as compared to MeOH-water and ACN-water systems. Sekulic et al.[5.53] also found that iso-eluotropic compositions of ACN-water and THF-water gave considerably lower retentions for amines. Since the transfer rules are developed for an "average solute", such deviations are not unusual. e.g. Bakalyar et al.[5.52] found that k' value of the last peak for THF-water system (k'=10.3) was much lower as compared as compared to MeOH-water system (k'=35.8)

(cf. Table 3.2). Similar deviations were also noted by d'Agostino et al.,where predicted iso-eluotropic THF-water composition provided 60%[5.35] and 46%[5.21] higher retentions than expected. It is important to note that in the latter cases, these compositions were used for optimisation by the MDS method regardless of the deviations noted.

In spite of the deviations observed in the present work, it is evident that almost all retentions were within the extended ideal k' range ($1<k'<20$). A further analysis of the k' values of the last retained peak shows an average of 15.1(Std.Dev.$\pm$3.46). This analysis indicates that although the specific interaction of acidic solutes with THF altered the predicted iso-eluotropic plane, it did not lead to a non-iso-eluotropic plane.

Once this solvent parameter space was defined, chromatograms other than Chrom/1,11,15, were obtained according to the two-factor five-level pseudo-factorial design as shown in Fig.5.4. The chromatograms with their COF value and solvent-systems used appear in Fig.5.9. It is evident that all chromatograms differ in their analysis time, total number of peaks and peak resolutions. It was also observed that 3-hydroxy- and 4-hydroxy-benzoic acid did not show any degree of separation under all conditions and therefore were considered as co-eluted components.

Now, in order to obtain a quantitative parameter for 'goodness' of the chromatogram COF values were calculated according to Eq.5.3 for all chromatograms, with following conditions.

1. The maximum number of peaks were eight (so number of pairs=k=7). Therefore, any chromatogram showing less than eight peaks (k<7) were penalised by adding an arbitrary value of -2.89 to COF.

2. Maximum acceptable analysis time (Tm) was set to 20 min. Any chromatogram showing higher analysis time (Tl) than Tm was penalised proportional to the excess time with a weighting factor(B) of 0.1.

3. Minimum acceptable peak-pair resolution (Rd) was set to 1.8.

4. All peak-pairs were given equal priority, i.e. A=1 for all pairs

In order to obtain a picture of the response surface the COF values according to the corresponding solvent compositions are shown in the solvent-parameter space (Fig.5.10(A)). It is apparent that the THF-end (C) of the plane shows worse COF values as compared to side AB. The maximum experimental COF was -1.80(Node2) and -2.11(Node12) was next best value. Intuitively, therefore, one might expect the optimum to be closer to these nodes.

As discussed earlier in Section 5.3 the bi-dimensional interpolation procedure requires a factorial arrangement of the data. After taking a reflection, a factorial-type matrix was obtained. This arrangement is shown in Fig.5.10(B) where the X-Y plane is the plane of reflection providing a symmetric matrix. Using this matrix the interpolation provides computed COF value at any desired solvent-composition, i.e. any combinations of A,B and C. But the known constraint is that the sum of A,B and C cannot exceed 100. For this reason the COF value of the compositions giving A+B+C>100 was set to -1e32, an extremely poor value for COF. Thus, although the imaginary plane was used for interpolation calculations it was precluded from the search for the optimum.

An up-hill simplex algorithm was used for locating global as well as local optima. If the search were done only once in the response plane, then there is a chance that the global optimum may go undetected (as may occur in sequential simplex optimisation). Therefore the algorithm was forced to search in the whole plane by providing starting values corresponding to each node. Since there are 15 nodes in a 2 factor 5 level design, total number of searches made were fifteen. A computer output of this search is given in Table 5.6. The end section of this table provides the starting compositions (Bi%, Ci%) from where the search was begun, calculated COF values(sorted) for the located optimum, if any, in that search and corresponding solvent compositions (B%, C%, A%) and

other parameters: {1} Rest.=Number of restart in the search, and {2} Itrs.=Number of iterations used in that search. Those rows containing 'Itrs.' values greater than the maximum number of iterations allowed can be ignored as the search was terminated while incomplete. There are three COF values, viz. -1.16, -1.79 and -2.86, where this condition was complied and therefore can be considered as optima in the decreasing order, -1.16 being the global optimum value of COF. These optima and corresponding predicted mobile phase compositions are given in Table 5.7. A three-dimensional view and contour-map of the response surface appear in Fig.5.11 and Fig.12 respectively, where the optima are marked with an arrow '->'. It is obvious that the graphical information provides an overview of the topography of the response plane and that the optima can be located with relative ease. But because graphical analysis does not provide accurate values of COF, they should be regarded as secondary data when compared to the numerical search like up-hill simplex.

Once the optima were located it was necessary to check the validity of these predictions experimentally. Experiments were performed employing the solvent compositions shown in Table 5.7 for all the three optima and a control. The solvent for control chromatogram was 100%A (30%MeOH-water). These chromatograms with their experimental COF values appear in Fig.5.13.

The control chromatogram compares very well with the previous chromatogram (Chrom/1, Fig.5.2) obtained under similar conditions. This comparison is reflected in their COF values, viz. COF(Chrom/1)=-3.97 and COF(Control)=-3.51. There is, however, a small difference due to experimental error. This comparison shows that the conditions for obtaining the previous 15 chromatograms were similar to those obtained for the predicted optima.

Now a comparison of predicted and experimental optimum solvent compositions can be done. It is obvious from Table 5.7 that the order of the optima (COF values) is same for the predicted and experimental data. Also it shows that the actual response values(COF) are quite similar, considering experimental error. It is always difficult to obtain an estimate of the variance of the response in such experiments as there are only a few unique values available, however the maximum difference between the predicted and experimental COF values was 0.4, as compared to the difference for the control experiment which was 0.46.

The preceding experimental results and discussion clearly suggests that the proposed optimisation strategy is able to locate global and local optima with sufficient accuracy.

Having established that that the proposed optimisation procedure was able to locate the optima with sufficient accuracy using a two-factor five-level pseudo-factorial design, it was, therefore, of interest to determine

whether it could provide acceptable optima with a smaller number of experiments i.e. a two-factor three-level design requiring six experiments (cf. Table 5.3) . This did not involve setting new experiments as a 3-level design is a sub-set of a 5-level design. Therefore, the necessary information was extracted from the previous experiments and an optimisation search was done on a 3x3 pseudo-factorial matrix. A computer output for this analysis is provided in Table 5.8 and the three-dimensional and contour view of the surface appear in Fig.5.14 and Fig.5.15 respectively.

A comparison of the outcome of the 3-level design (Table 5.8) with that of the 5-level (Table 5.6) reveals that the predicted optimum for 3-level is qualitatively as well as quantitavely different from the 5-level design. Also it is noticable that the COF value is low (-2.288) as compared to 5-level design(-1.16). Neverthless, the optimum predicted from the 3-level design is better than the second optimun (Optimum II) of the 5 level experiments. This is not apparent if one compares the COF values directly. Consider the solvent composition corresponding to Optimum II (of the 5 level design), which is 23.02/0.00/76.97 (A/B/C). Now consider similar composition in Table 5.8 for 3-level experiment, the composition corresponding to COF=-2.650, which is 23.15/0.00/76.85 (A/B/C) which may be considered, not correctly but for reasoning, the second optimum for 3-level design. Thus if the second optima for 3- as

well as 5-level experiments are similar then the optimum predicted from a three level design is atleast somewhere between the global (Optimum I) and the second optimum (Optimum II) for five level design. Therefore, it can be correctly assumed that although the three-level design is not able to provide an optimum as good (or accurate) as five-level design, which is obvious, it is able to provide a good estimate of the global optimum.

Once the validity of the proposed approach for optimisation has been checked on the experimental data of this study, it was decided to examine the data from the literature. But because experimental data similar to a 5 level design were not available data from those reports employing mixture design statistical (Simplex, not sequential simplex) procedure was used. This did not pose any difficulty as it was shown earlier that the the proposed optimisation strategy is easily adaptable for different needs (but for only factorial or pseudo-factorial designs). The data were taken from reports by Glajch et al.[5.15], Smith et al.[5.58] and d'Agostino et al.[5.21] and will be called Data1, Data2 and Data3 respectively. Table 5.9, 5.10 and 5.11 contains the experimental data and computer output as given by the programme "ORIENT". An overall analysis of the data in Table 5.9,5.10 and 5.11 indicates that the qualitative compositions predicted by ORIENT, in this work, are exactly the same as the reported solvent-systems. However, the difference is visible in the quantitative comparison. A comparison of COF

values (not reported for Data2) shows close agreement for Data1 but a significant difference for Data3. The reason for this quantitative difference is not surprising, as all the reports (Data1,Data2 and Data3) used a seven point simplex design out of which only six points are identical with the design of the present study. Therefore, the difference observed in the predicted optima is due to the seventh extra point incorporated in the modelling reported. This could influence the topography of the response surface, hence provide different COF values implying different solvent compositions for optima. If this point was excluded from the modelling in the reported data, then the predicted optima are expected to be quite similar to those predicted by the methodology employed in the present work.

This view is further supported by a comparison of the contour maps created in this work and those reported in the literature. They appear in Fig.5.16,5.17 and 5.18 for Data1, Data2 and Data3 respectively. The optima located by 'ORIENT' are shown by '$' and with '->' for those reported. A general similarity is obtained. It is also obvious that the locations of the optima are not very different. This comparison clearly supports the view stated earlier that incorporation of the seventh extra point in the models developed in the reports does alter the topography of the response plane, although not significantly. It will be shown later(section 5.5.3) using simulation that for Data3 the optimum predicted by 'ORIENT' is providing

simulated retentions which shows close agreement with the simulated retentions for the reported optimum.

5.5.2 Overlapping Resolution Mapping (ORM)

The discussion so far has concerned the optimisation of selectivity using a single value parameter or composite criterion (COF). It has also been shown that this approach does provide satisfactory optima using the 'ORIENT' programme. However, the risks of using composite criterion are obvious as discussed in Section 5.2.2. This is especially true for those cases where cross-over of the retentions of the peaks is likely, which is often the case when the sample matrix is large and contains solutes from distinct chemical classes. In such situations the merit of the Overlapping Resolution Mapping (ORM) can be exploited for more reliable optimisation. Therefore, it was of interest to study if the proposed strategy may be modified for ORM-type optimisation.

The first step for ORM-type optimisation was to calculate the resolution(Rs) between the peaks of each pair for all chromatograms obtained according to the 2-factor 5-level design. There were seven pairs of peaks and a list of their Rs values corresponding to the solvent-system used appear in Table 5.12. It should be noted that a value of 2.0 was assigned to those pairs showing Rs either equal or greater than two.

From the data given in Table 5.12 a 5x5 pseudo-factorial matrix containing Rs values was created for each pair. This matrix was then used for interpolation and response(Rs) surface modelling so that a contour map of resolution can be obtained for each peak-pair. The resolution maps for seven pairs appear in Fig.5.19(1)-5.19(7). It is important to note that the symbols used for each contour level are relative, hence a direct comparison, according to symbol should not be attempted between two maps. Therefore Rs value related with each symbol are provided in each map separately.

It evident, from a comparison of the seven resolution maps, that pair 1,2,3,5 and 6 show some area of Rs greater than 1.8, indicating that satisfactory separations of these pairs is possible in some part(s) of the iso-eluotropic plane (ABC). In the case of pair 4 this area is extremely small whereas for pair 7 there is no such area (Rs(maximum for pair 7) < 1.0). Therefore, due to pair 4 and 7,it is obvious that there cannot be an optimum where all the peaks are satisfactorily(Rs>1.8) resolved. But as the definition of the the optimum implies a region of the best possible separation could be located. Now in order to translate the intuitive meaning of 'best possible' to a quantitative one, the folowing assumptions were made:

1. All regions of the solvent-parameter space showing Rs>1.8 for a given peak-pair should be considered as satisfactory, and

2. For those pairs which do not comply with condition (1) the regions showing top 20% separation for the given pair should be considered satisfactory. For example pair 7 has Rs(maximum)=0.93. This Rs(max) can be considered as 100% and any region showing a separation for this pair between 80-100% (i.e. Rs between 0.744 to 0.93) should be considered as satisfactory.

On the basis of the above criteria a physical comparison of all the resolution maps (by overlapping) can be done. However, this procedure is somewhat difficult and does not provide an objective means of comparison. In the present work a different numerical approach was developed such that an objective judgement can be made from the comparison of resolution maps. It should be mentioned that since this is a computer based numerical approach it can be iteratively used for re evaluation of the same resolution maps but subjected to different criteria of satisfactory resolution. The basic steps of this procedure,called "NUMEROGRAPH", appear below and a listing of the programme can be found in Appendix C.

1. A nxn matrix (n=25) was created where each cell corresponds to a 4% change in the solvent composition either down (for solvent C) or across (for solvent B) the matrix.

2. Initially all cells were given a value i (i=7 for seven pairs for this study). This provides an ideal matrix where all pairs(7) are satisfactorily (subject to the criteria discussed earlier) resolved in the entire solvent-parameter space defined by A,B and C.

3. The resolution response surface for each pair was evaluated at compositions corresponding to each cell. For example, at matrix cell X(5,7) would correspond to 5x4=20% of B and 7x4=28% of C the Rs value for each pair was calculated using bi-dimensional polynomial interpolation.

4. The calculated Rs values were checked against the criteria (1) and (2) as discussed earlier. If the response is <u>not</u> satisfactory then a value of 1.00 was removed from the corresponding cell, i.e. the pair did not resolve at that composition. Thus the regions providing satisfactory resolutions would have a higher value as compared to the rest of the area.

5. For each peak-pair process (3) and (4) are repeated.

6. The final matrix, therefore,would contain values from 0 to 7 depending upon how many peak-pairs were separated at each cell.

The matrices, here called "Numerograms", generated appear in Fig.5.20 for 1 to 7 pairs. It is apparent that the values remaining in each cell varies from cell

to cell and from matrix to matrix. The final numerogram, or matrix, then shows how many pairs were resolved (subject to criteria (1) and (2)). Those cells with maximum values (6) are circled. Thus there are 6 out of 7 pairs showing resolution at the compositions corresponding to the cells circled. The solvent composition for optima predicted by "NUMEROGRAPH" programme appear in Table 5.13.

A comparison of the optima predicted by the ORM-type approach (Table 5.13) with COF based optima (Table 5.7) shows differences both in qualitative as well as quantitative compositions.

5.5.3 Chromatogram Simulation

The optimisation methodology considered so far were based on all-peak composite (COF) or two-peak composite (Rs, in ORM) criteria. However it would be ideal if retention of each peak (k') and its characteristics, such as width(w) and symmetry, can be predicted so that optimisation may be achieved on the basis of one-peak criterion. In other words, chromatograms can be simulated at the desired solvent-system and the goodness of the chromatogram can be judged directly. However, one of the most difficult problem would be to collect information on individual peaks, especially when the peaks are partially resolved. Seaton et al.[5.55] and Vandeginste et al.[5.56] have shown that deconvolution of such partially resolved peaks may be achieved employing multichannel detection and using

multivariate analysis technique such as Iterative Target Testing Transformation (ITTT). In spite of such problems attempts have been made[5.15,5.35] for producing simulated chromatogram, or so called "synthetic chromatogram", on the basis of the retentions (k') only.

It was, therefore, of interest to investigate if the numerical algorithm, used for the optimisation strategy proposed in this work, could find use in the chromatogram simulation.

Therefore, the algorithm was modified ("SIMULA") for simulation, which is listed in Appendix D. A pseudo-factorial 2-factor 5-level design matrix of retention (k', Table 5.5) of six peaks was used for the simulation purpose. Using this as the data base the retentions of the same peaks were predicted for three optima (I, II and III), a control and four test compositions. The predicted and experimental k' values appear in Table 5.14.

The obvious observation is that the maximum error is $\pm$10.18% or less. It is also evident that the experimental data shows a close agreement with the retentions predicted by "SIMULA" (n=48, r=0.997, s=0.358). A graphical comparison is also provided in Fig.5.21. Such a good agreement between the experimental and predicted retention is not unexpected as the variation of k' with change in solvent composition is a simple and smooth function as compared to Rs or COF which shows higher degree of complexities

(as they are composite criteria).

An attempt was also made to check if this programme is also able to provide correct predictions when applied to the literature data. The data(derived from the published plot) for this test were taken from d'Agostino et al.[5.21]. The k' values for ten steroids is given in Table 5.15. Pseudo-factorial matrices for all ten peaks were provided to "SIMULA" for predicting retentions for (1) a reported test composition, (2) reported optimum, and (3) for the optimum predicted by 'ORIENT' (cf. Table 5.11). A comparison of the predicted and experimental k' is exhibited in Table 5.16 and graphical comparison is given in Fig.5.22. It is apparent the data compare well with the experimental values as the maximum %error is within $\pm$11.32% with a high degree of correlation (n=20, r=0.983, s=0.663). A comparison of retentions for the reported optimum with that of the retentions for optimum predicted by 'ORIENT' (Table 5.16) shows close agreement. This finding also support the previously stated view(section 5.5.1) that the optimum predicted by 'ORIENT' using only a six point experiment (2-factor 3-level design) are valid. This analysis provides preliminary results which are encouraging for wider application.

Although this attempt is far removed from simulating the whole chromatograms it would be reasonable to accept that the numerical algorithm, used in this work for optimisation, may also be used in simulation

studies.

5.6 CONCLUSIONS

On the basis of the experimental analysis, data analysis and the principles employed in the proposed optimisation strategy, the following conclusions can be drawn:

1. Relatively precise predictions of the solvent compositions providing global as well as local optima are possible for composite optimisation criterion(COF).

2. It has been demonstrated that objective prediction of the optima may be achieved by Overlapping Resolution Mapping with the methodology employed in this work.

3. Use of the numerical method has been found useful for the prediction of retention and shows potential for chromatogram simulation studies.

4. The experimental design employed is flexible and can be varied on the basis of the complexity of the sample matrix.

5. The modified sequential simplex procedure has been found to be efficient in searching for optima and provides more accurate estimates of solvent compositions than the grid search method.

TABLE 5.1

A LIST OF AVAILABLE OPTIMISATION PROCEDURES, NUMBER OF EXPERIMENTS REQUIRED AND THE TYPE OF MODEL EMPLOYED

METHOD	EXPERIMENTS	MODEL¤	REFERENCE
WINDOW DIAGRAM	2 to 9	E/CM/LI	5.1-5.8
CRITICAL BAND	2	L/E	5.9,5.10
FULL FACTORIAL	4 to 42	SE/CM	5.11-5.14,5.19
SIMPLEX LATTICE	7	E/V	5.15-5.17
EXTENDED LATTICE	10	E	5.18
LIMITED FACTORIAL	15	SE	5.20
MODIFIED LATTICE	12	E	5.21
QUADRATIC DESIGN	Variable	E	5.22
ITERATIVE DESIGN	4 to 10	LI	5.23-5.27
SEQUENTIAL GLOBAL	Variable	MLS	5.28

¤ Where, E = Empirical
SE= Semi-empirical
CM= Chromatographic model
LI= Linear interpolation
L = Linear
V = Visual comparison
MLS= Moving least squares

TABLE 5.2

COMPARISON OF STOCHASTIC AND HEURISTIC APPROACHES.

STOCHASTIC	HEURISTIC
Strategic and simultaneous.	Non-strategic and sequential.
Fixed number of experiments.	Experiments variable.
Finds global optimum.	Global optimum not guaranteed
Empirical function provided.	Does not provide a function.
Complicated for automation.	Easy to automate.
Error estimate on predictions.	No such estimate possible.
Experiments increases rapidly.	Increase is not so rapid.

TABLE 5.3

LEVELS OF ORGANIC MODIFIER AND NUMBER OF EXPERIMENTS.

LEVELS						n	E	P
1	2	3	4	5	6			
0	100.0					2	3	1
0	50.0	100.0				3	6	2
0	33.3	66.7	100.0			4	10	3
0	25.0	50.0	75.0	100.0		5	15	4
0	20.0	40.0	60.0	80.0	100.0	6	21	5

Where,
n = Levels of mixing,
E = Number of experiments required; n(n+1)/2,
P = Degree of polynomial fitted; (n-1).

TABLE 5.4

COMPOUNDS USED FOR ANALYSIS AND THEIR CODES.

COMPOUND	CODE
4-amino benzoic acid	4NH2
3-hydroxy benzoic acid	3OH
4-hydroxy benzoic acid	4OH
2-nitro benzoic acid	2NO2
2-amino benzoic acid	2NH2
3-nitro benzoic acid	3NO2
4-nitro benzoic acid	4NO2
Phenyl acetic acid	PAA

TABLE 5.5

AVERAGE k' VALUES OF SUBSTITUTED BENZOIC ACIDS ANALYSED UNDER DIFFERENT SOLVENT SYSTEMS.

A%	B%	C%	4NH2	4/3OH*	2NO2	2NH2	PAA	3NO2	4NO2	COF
100	0	0	1.03	2.06	3.84	3.84	7.46	9.50	10.62	-3.97
75	25	0	1.22	2.25	2.98	4.42	8.69	11.24	12.47	-1.80
75	0	25	1.32	3.41	5.14	5.14	7.08	13.77	15.26	-3.10
50	50	0	1.28	2.19	3.69	4.74	9.36	12.30	13.62	-2.33
50	25	25	1.39	3.19	5.20	5.20	7.48	13.55	15.01	-4.13
50	0	50	1.54	4.17	5.99	5.99	6.98	16.92	18.42	-3.17
25	75	0	1.24	1.82	3.83	4.28	8.46	11.20	12.42	-2.49
25	50	25	1.52	3.03	4.05	5.41	8.08	13.72	15.20	-4.03
25	25	50	1.46	3.62	5.59	5.59	6.86	15.02	16.50	-4.82
25	0	75	1.61	4.46	6.36	6.36	17.63	18.67	20.28	-7.35
0	100	0	1.07	1.31	3.25	3.25	6.17	08.14	09.02	-5.20
0	75	25	1.34	2.40	3.86	4.53	7.00	11.41	12.62	-2.11
0	50	50	1.41	3.03	3.91	4.97	6.39	12.84	14.12	-4.67
0	25	75	1.77	4.28	4.93	6.51	16.71	17.80	19.51	-5.17
0	0	100	1.69	4.63	6.65	6.65	17.74	19.43	21.41	-5.70

* Co-elution of 3OH- and 4OH-Benzoic Acid

TABLE 5.6

COF DATA MATRIX FOR 2-FACTOR 5-LEVEL EXPERIMENT AND COMPUTER OUTPUT RESULTING FROM SIMPLEX SEARCH FOR THE PRESENT WORK

B% I / I / V	>----------C%----------> 0.00	25.00	50.00	75.00	100.00
0.00	-3.97	-3.10	-3.71	-7.35	-5.70
25.00	-1.80	-4.13	-4.82	-5.17	-7.35
50.00	-2.33	-4.03	-4.67	-4.82	-3.71
75.00	-2.49	-2.11	-4.03	-4.13	-3.10
100.00	-5.20	-2.49	-2.33	-1.80	-3.97

Maximum iterations = 1000
Tolerance for step change = 0.00001000
Maximum step size = 100.

Bi%	Ci%	COF(Cal)	B%	C%	A%	Rest.	Itrs.
0.00	0.00	-2.10164452	12.79	0.00	87.21	67	1010
0.00	25.00	-1.16398287	84.99	15.01	-0.00	5	90
0.00	50.00	-2.85663915	0.00	34.21	65.79	23	473
0.00	75.00	-4.72263956	18.48	50.32	31.20	71	1021
0.00	100.00	-1.16398323	84.98	15.01	0.01	19	322
25.00	0.00	-1.79441285	24.05	0.00	75.95	59	1007
25.00	25.00	-1.84357572	28.36	0.00	71.64	63	1020
25.00	50.00	-1.16398478	84.99	15.00	0.01	14	209
25.00	75.00	-1.25621939	81.38	11.87	6.75	64	1009
50.00	0.00	-1.16398335	84.99	15.01	0.00	4	98
50.00	25.00	-1.34899437	79.68	10.62	9.70	65	1013
50.00	50.00	-1.16398311	84.97	15.01	0.02	4	94
75.00	0.00	-1.29447472	79.68	13.24	7.08	63	1014
75.00	25.00	-1.79234922	23.02	0.00	76.97	13	239
100.00	0.00	-1.16398394	84.97	15.01	0.02	9	142

Sorted COF values and corresponding data

0.00	25.00	-1.16398287	84.99	15.01	-0.00	5.	90.
50.00	50.00	-1.16398311	84.97	15.01	0.02	4.	94.
0.00	100.00	-1.16398323	84.98	15.01	0.01	19.	322.
50.00	0.00	-1.16398335	84.99	15.01	0.00	4.	98.
100.00	0.00	-1.16398394	84.97	15.01	0.02	9.	142.
25.00	50.00	-1.16398478	84.99	15.00	0.01	14.	209.
25.00	75.00	-1.25621939	81.38	11.87	6.75	64.	1009.
75.00	0.00	-1.29447472	79.68	13.24	7.08	63.	1014.
50.00	25.00	-1.34899437	79.68	10.62	9.70	65.	1013.
75.00	25.00	-1.79234922	23.02	0.00	76.97	13.	239.
25.00	0.00	-1.79441285	24.05	0.00	75.95	59.	1007.
25.00	25.00	-1.84357572	28.36	0.00	71.64	63.	1020.
0.00	0.00	-2.10164452	12.79	0.00	87.21	67.	1010.
0.00	50.00	-2.85663915	0.00	34.21	65.79	23.	473.
0.00	75.00	-4.72263956	18.48	50.32	31.20	71.	1021.

Where, Bi and Ci are initial composition for search,
Rest. = Number of restart made during the search, and
Itrs. = Number of iterations required for that search

TABLE 5.7

COMPARISON OF THE PREDICTED AND EXPERIMENTAL OPTIMA

		Solvent¤			COF	
Optimum	Type	A%	B%	C%	Predicted	Experimental
I	Global	0.0	85.0	15.0	-1.16	-1.33
II	Local	77.0	23.0	0.0	-1.79	-1.39
III	Local	65.7	0.0	34.3	-2.86	-2.75
Control	-	100.0	0.0	0.0	-3.97§	-3.51

¤ Values rounded to first decimal point.
§ Previous experimental value (see Chrom/1)

TABLE 5.8

COF DATA MATRIX FOR 2-FACTOR 3-LEVEL EXPERIMENT AND COMPUTER OUTPUT RESULTING FROM SIMPLEX SEARCH FOR THE PRESENT WORK

B% I / I / V	>----------C%----------> 0.00	50.00	100.00
0.00	-3.97	-3.71	-5.70
50.00	-2.33	-4.67	-3.71
100.00	-5.20	-2.33	-3.97

Maximum iterations = 1000
Tolerance for step change = 0.00001000
Maximum step size = 100.

Bi%	Ci%	COF(Cal)	B%	C%	A%	Rest.	Itrs.
0.00	0.00	-2.65017962	23.15	0.00	76.85	63	1004
0.00	50.00	-3.54470468	0.00	32.18	67.82	56	1009
0.00	100.00	-2.28826594	43.16	0.00	76.84	35	597
50.00	0.00	-2.28820491	43.17	0.00	56.83	30	488
50.00	50.00	-3.13078260	65.51	7.74	26.74	61	1008
100.00	0.00	-2.28826594	43.17	0.00	56.83	35	598

Sorted COF values and corresponding data

50.00	0.00	-2.28820491	43.17	0.00	56.83	30.	488.
0.00	100.00	-2.28826594	43.16	0.00	56.84	35.	597.
100.00	0.00	-2.28826594	43.17	0.00	56.83	35.	598.
0.00	0.00	-2.65017962	23.15	0.00	76.85	63.	1004.
50.00	50.00	-3.13078260	65.51	7.74	26.74	61.	1008.
0.00	50.00	-3.54470468	0.00	32.18	67.82	56.	1009.

Where, Bi and Ci are initial composition for search,
Rest. = Number of restart made during the search, and
Itrs. = Number of iterations required for that search

TABLE 5.9

COF DATA MATRIX FOR 2-FACTOR 3-LEVEL EXPERIMENT AND COMPUTER OUTPUT RESULTING FROM SIMPLEX SEARCH FOR 'DATA 1'

Solvent system			
A%	B%	C%	COF (Experimental)
100	0	0	-3.21
50	50	0	-2.89
0	100	0	-1.80
0	50	50	-0.02
0	0	100	-5.78
50	0	50	-3.41
33.3	33.3	33.3	-0.38
0	61	39	-0.07 Reported optimum.
0	52	48	-0.01 Calculated by ORIENT

A = 63% MeOH
B = 52% ACN
C = 39% THF

B% I V	C% 0.00	C% 50.00	C% 100.00
0.00	-3.21	-3.41	-5.78
50.00	-2.89	-0.02	-3.41
100.00	-1.80	-2.89	-3.21

Maximum iterations = 2000
Tolerance for step change = 0.00001000
Maximum step size = 100.

Bi%	Ci%	COF(Cal)	B%	C%	A%	Rest.	Itrs.
0.00	0.00	-2.19353247	16.34	15.55	68.11	130	2003
0.00	50.00	-0.00957824	52.01	47.98	0.00	5	113
0.00	100.00	-0.00957822	52.02	47.99	-0.00	24	494
50.00	0.00	-0.38452142	52.65	30.58	16.77	134	2009
50.00	50.00	-0.00957824	52.01	47.99	0.00	2	72
100.00	0.00	-1.79999995	100.00	0.00	0.00	0	17

Sorted COF values and corresponding data

0.00	100.00	-0.00957822	52.02	47.99	-0.00	24.	494.
0.00	50.00	-0.00957824	52.01	47.98	0.00	5.	113.
50.00	50.00	-0.00957824	52.01	47.99	0.00	2.	72.
50.00	0.00	-0.38452142	52.65	30.58	16.77	134.	2009.
100.00	0.00	-1.79999995	100.00	0.00	0.00	0.	17.
0.00	0.00	-2.19353247	16.34	15.55	68.11	130.	2003.

Where, Bi and Ci are initial composition for search,
Rest. = Number of restart made during the search, and
Itrs. = Number of iterations required for that search

TABLE 5.10

COF DATA MATRIX FOR 2-FACTOR 3-LEVEL EXPERIMENT AND COMPUTER OUTPUT RESULTING FROM SIMPLEX SEARCH FOR 'DATA 2'

Solvent system			
A%	B%	C%	COF (Experimental)
100	0	0	-0.36
50	50	0	0.00
0	100	0	-0.19
0	50	50	-2.00
0	0	100	-1.00
50	0	50	-1.72
33.3	33.3	33.3	-4.37
30	70	00	* Reported optimum.
42	58	00	0.0068 Calculated by ORIENT

A = 41% MeOH
B = 30% ACN
C = 28% THF

* Qualitative from contour map.

B% I / I / V	C% 0.00	C% 50.00	C% 100.00
0.00	-0.36	-1.72	-1.00
50.00	0.00	-2.00	-1.72
100.00	-0.19	0.00	-0.36

Maximum iterations = 5000
Tolerance for step change = 0.00001000
Maximum step size = 100.

Bi%	Ci%	COF(Cal)	B%	C%	A%	Rest.	Itrs.
0.00	0.00	-0.30678082	4.63	0.00	95.37	333	5012
0.00	50.00	-0.21960434	12.64	0.00	87.36	318	5002
0.00	100.00	0.00687743	100.00	42.15	-42.15	7	238
50.00	0.00	0.00687755	57.88	0.00	42.12	133	2576
50.00	50.00	0.00687756	57.89	0.00	42.11	248	4520
100.00	0.00	-0.06708656	100.00	16.29	-16.29	305	5014

Sorted COF values and corresponding data

50.00	50.00	0.00687756	57.89	0.00	42.11	248.	4520.
50.00	0.00	0.00687755	57.88	0.00	42.12	133.	2576.
0.00	100.00	0.00687743	100.00	42.15	-42.15	7.	238.
100.00	0.00	-0.06708656	100.00	16.29	-16.29	305.	5014.
0.00	50.00	-0.21960434	12.64	0.00	87.36	318.	5002.
0.00	0.00	-0.30678082	4.63	0.00	95.37	333.	5012.

Where, Bi and Ci are initial composition for search,
Rest. = Number of restart made during the search, and
Itrs. = Number of iterations required for that search

TABLE 5.11

COF DATA MATRIX FOR 2-FACTOR 3-LEVEL EXPERIMENT AND COMPUTER OUTPUT RESULTING FROM SIMPLEX SEARCH FOR 'DATA 3'

Solvent system				
A%	B%	C%	COF (Experimental)	
100	0	0	-11.26	
50	50	0	-6.67	
0	100	0	-4.59	
0	50	50	-6.49	
0	0	100	-8.77	
50	0	50	-1.92	
33.3	33.3	33.3	-3.88	
64	0	36	-0.85	Reported optimum.
46	0	54	-1.87	Calculated by ORIENT

A = 35% MeOH
B = 20% ACN
C = 12% THF

B% I V	C% 0.00	C% 50.00	C% 100.00
0.00	-11.26	-1.92	-8.77
50.00	-6.67	-6.49	-1.92
100.00	-4.59	-6.67	-11.26

Maximum iterations = 5000
Tolerance for step change = 0.00001000
Maximum step size = 100.

Bi%	Ci%	COF(Cal)	B%	C%	A%	Rest.	Itrs.
0.00	0.00	-1.87213099	0.00	53.86	46.14	177	3126
0.00	50.00	-1.87213361	0.00	53.82	46.18	125	2257
0.00	100.00	-2.31141281	0.11	65.38	34.51	331	5012
50.00	0.00	-4.59025383	99.99	0.00	0.01	8	187
50.00	50.00	-2.26648331	0.00	64.88	35.12	322	5010
100.00	0.00	-4.59000015	100.00	0.00	0.00	0	21

Sorted COF values and corresponding data

0.00	0.00	-1.87213099	0.00	53.86	46.14	177.	3126.
0.00	50.00	-1.87213361	0.00	53.82	46.18	125.	2257.
50.00	50.00	-2.26648331	0.00	64.88	35.12	322.	5010.
0.00	100.00	-2.31141281	0.11	65.38	34.51	331.	5012.
100.00	0.00	-4.59000015	100.00	0.00	0.00	0.	21.
50.00	0.00	-4.59025383	99.99	0.00	0.01	8.	187.

Where, Bi and Ci are initial composition for search,
Rest. = Number of restart made during the search, and
Itrs. = Number of iterations required for that search

TABLE 5.12

RESOLUTION VALUES OF SEVEN PEAK-PAIRS OBTAINED BY DIFFERENT SOLVENT COMPOSITIONS.

Chrom.§	Solvent			Pair Number						
	A%	B%	C%	1	2	3	4	5	6	7
1	100	0	0	2.00¤	1.36	1.21	0.10	2.00	2.00	0.81
2	75	25	0	2.00	1.38	0.83	1.41	2.00	2.00	0.89
3	75	0	25	2.00	0.68	1.76	0.53	2.00	2.00	0.76
4	50	50	0	2.00	2.00	0.53	1.12	2.00	2.00	0.79
5	50	25	25	2.00	1.79	1.15	0.10	2.00	2.00	0.81
6	50	0	50	2.00	0.60	1.88	0.76	1.29	2.00	0.73
7	25	75	0	1.65	1.28	0.99	0.73	2.00	2.00	0.73
8	25	50	25	2.00	1.27	0.10	1.71	2.00	2.00	0.78
9	25	25	50	2.00	1.22	1.16	0.10	1.58	2.00	0.88
10	25	0	75	2.00	1.30	0.71	0.10	2.00	0.77	0.38
11	0	100	0	0.40	1.46	2.00	0.10	2.00	2.00	0.49
12	0	75	25	1.13	1.48	2.00	0.81	2.00	2.00	0.70
13	0	50	50	2.00	1.13	0.10	1.23	1.80	2.00	0.71
14	0	25	75	2.00	0.69	0.63	0.91	2.00	0.59	0.72
15	0	0	0	2.00	0.51	0.57	1.06	2.00	0.87	0.78

§ Chrom., Chromatogram number,
¤ Resolution(Rs) 2.0 or greater.

TABLE 5.13

OPTIMUM SOLVENT COMPOSITIONS AS PREDICTED BY 'NUMEROGRAPH' PROGRAMME EMPLOYING OVERLAPPING RESOLUTION MAPPING

	Solvent-system		
Optimum	A%	B%	C%
1	56	16	28
2	52	40	8
3	48	44	8
4	44	48	8

TABLE 5.14

COMPARISON OF EXPERIMENTAL k' WITH THOSE VALUES PREDICTED USING SIMULA PROGRAMME AT THE PREDICTED OPTIMUM COMPOSITIONS (I, II, III) AND OTHER TEST COMPOSITIONS FOR SIX MAJOR PEAKS.

Peak	B%	C%	k'(Exptl)	k'(Pred)	Error	%Error
			Optimum I			
1.	85.00	15.00	1.13	1.19	-0.060	-5.31
2.	85.00	15.00	1.73	1.84	-0.110	-6.36
3.	85.00	15.00	3.83	3.44	0.390	10.18
4.	85.00	15.00	3.60	3.85	-0.250	-6.94
5.	85.00	15.00	9.21	9.28	-0.070	-0.76
6.	85.00	15.00	10.17	10.25	-0.080	-0.79
			Optimum II			
1.	23.00	0.00	1.21	1.28	-0.070	-5.79
2.	23.00	0.00	2.24	2.28	-0.040	-1.79
3.	23.00	0.00	2.95	2.94	0.010	0.34
4.	23.00	0.00	4.37	4.48	-0.110	-2.52
5.	23.00	0.00	11.09	10.93	0.160	1.44
6.	23.00	0.00	12.31	12.15	0.160	1.30
			Optimum III			
1.	0.00	34.30	1.42	1.33	0.090	6.34
2.	0.00	34.30	3.76	3.47	0.290	7.71
3.	0.00	34.30	5.52	4.99	0.530	9.60
4.	0.00	34.30	5.52	4.99	0.530	9.60
5.	0.00	34.30	15.10	14.13	0.970	6.42
6.	0.00	34.30	16.61	15.51	1.100	6.62
			Control			
1.	0.00	0.00	1.03	1.13	-0.100	-9.71
2.	0.00	0.00	2.06	2.21	-0.150	-7.28
3.	0.00	0.00	3.84	4.16	-0.320	-8.33
4.	0.00	0.00	3.84	4.16	-0.320	-8.33
5.	0.00	0.00	9.50	10.06	-0.560	-5.89
6.	0.00	0.00	10.62	11.16	-0.540	-5.08

TABLE 5.14 (Continued)

COMPARISON OF EXPERIMENTAL k' WITH THOSE VALUES PREDICTED USING SIMULA PROGRAMME AT THE PREDICTED OPTIMUM COMPOSITIONS (I, II, III) AND OTHER TEST COMPOSITIONS FOR SIX MAJOR PEAKS.

Peak	B%	C%	k'(Exptl)	k'(Pred)	Error	%Error
			Test Composition 1			
1.	32.60	0.00	1.25	1.27	-0.020	-1.60
2.	32.60	0.00	2.27	2.19	0.080	3.52
3.	32.60	0.00	3.17	3.13	0.040	1.26
4.	32.60	0.00	4.58	4.50	0.080	1.75
5.	32.60	0.00	11.73	11.09	0.640	5.46
6.	32.60	0.00	13.00	12.31	0.690	5.31
			Test Composition 2			
1.	43.20	0.00	1.27	1.22	0.050	3.94
2.	43.20	0.00	2.24	2.06	0.180	8.04
3.	43.20	0.00	3.50	3.37	0.130	3.71
4.	43.20	0.00	4.72	4.47	0.250	5.30
5.	43.20	0.00	12.19	11.18	1.010	8.29
6.	43.20	0.00	13.50	12.30	1.200	8.89
			Test Composition 3			
1.	33.90	0.00	1.25	1.23	0.020	1.60
2.	33.90	0.00	2.27	2.12	0.150	6.61
3.	33.90	0.00	3.21	3.17	0.040	1.25
4.	33.90	0.00	4.61	4.47	0.140	3.04
5.	33.90	0.00	11.81	11.12	0.690	5.84
6.	33.90	0.00	13.08	12.25	0.830	6.35
			Test Composition 4			
1.	64.10	35.90	1.48	1.43	0.050	3.38
2.	64.10	35.90	2.86	2.67	0.190	6.64
3.	64.10	35.90	3.56	3.48	0.080	2.25
4.	64.10	35.90	5.08	4.79	0.290	5.71
5.	64.10	35.90	12.79	11.74	1.050	8.21
6.	64.10	35.90	14.13	12.87	1.260	8.92

TABLE 5.15

RETENTION DATA k' AS OBTAINED FROM ref.[5.21]

	Solvent proportions A/B/C§							Optimum¤
	100/0/0	0/100/0	0/0/100	50/50/0	0/50/50	50/0/50	33/33/33	54/0/36
1¶	09.46	10.87	08.05	11.90	07.88	07.53	08.09	07.88
2	10.87	12.15	08.35	13.86	08.39	07.96	08.47	08.60
3	13.14	12.58	08.86	16.65	09.07	08.30	09.84	09.67
4	13.99	13.39	10.31	17.03	09.42	09.37	09.93	10.53
5	15.70	13.69	11.77	17.29	10.31	10.78	10.66	12.41
6	18.23	15.41	12.97	18.14	10.70	11.73	11.68	13.44
7	19.21	15.88	13.09	20.92	10.87	12.50	12.37	14.34
8	19.21	17.20	17.20	22.42	12.75	14.21	14.38	15.66
9	22.72	23.23	19.17	24.13	15.53	15.06	15.45	17.63
10	22.72	24.39	27.13	26.10	18.70	19.55	19.34	21.82

§ A=MeOH-Water; 35/65
B=ACN -Water; 20/80
C=THF -Water; 12/88

¤ Optimum as reported in ref.[5.21]

¶ Peak number corresponding to a steroid (cf. [5.21])

TABLE 5.16

COMPARISON OF THE EXPERIMENTAL AND PREDICTED (BY 'SIMULA') RETENTIONS k' FOR DATA FROM TABLE 5.15

Peak	B%	C%	k'(Exptl)	k'(Pred)	Error	%Error
			Reported Test Composition			
1.	33.33	33.33	8.09	8.26	-0.17	-2.10
2.	33.33	33.33	8.47	8.94	0.02	0.24
3.	33.33	33.33	9.84	9.91	-0.07	-0.71
4.	33.33	33.33	9.93	10.33	-0.40	-4.03
5.	33.33	33.33	10.66	11.26	-0.60	-5.63
6.	33.33	33.33	11.68	11.82	-0.15	-1.28
7.	33.33	33.33	12.37	12.26	0.01	0.08
8.	33.33	33.33	14.38	14.02	0.35	2.43
9.	33.33	33.33	15.45	16.50	-1.05	-6.80
10.	33.33	33.33	19.34	19.41	-0.07	-0.36
			Reported Optimum Composition			
1.	00.00	36.00	7.88	7.82	0.06	0.76
2.	00.00	36.00	8.60	8.44	0.16	1.86
3.	00.00	36.00	9.67	9.11	0.56	5.79
4.	00.00	36.00	10.53	10.10	0.43	4.08
5.	00.00	36.00	12.41	11.57	0.84	6.77
6.	00.00	36.00	13.44	12.77	0.67	4.99
7.	00.00	36.00	14.34	13.64	0.67	4.21
8.	00.00	36.00	15.66	14.80	0.86	5.49
9.	00.00	36.00	17.63	16.02	1.61	9.13
10.	00.00	36.00	21.82	19.35	2.47	11.32
			Optimum Predicted by ORIENT programme			
1.	00.00	53.86	-	7.48	-	-
2.	00.00	53.86	-	7.87	-	-
3.	00.00	53.86	-	8.15	-	-
4.	00.00	53.86	-	9.24	-	-
5.	00.00	53.86	-	10.64	-	-
6.	00.00	53.86	-	11.55	-	-
7.	00.00	53.86	-	12.28	-	-
8.	00.00	53.86	-	14.16	-	-
9.	00.00	53.86	-	14.96	-	-
10.	00.00	53.86	-	19.75	-	-

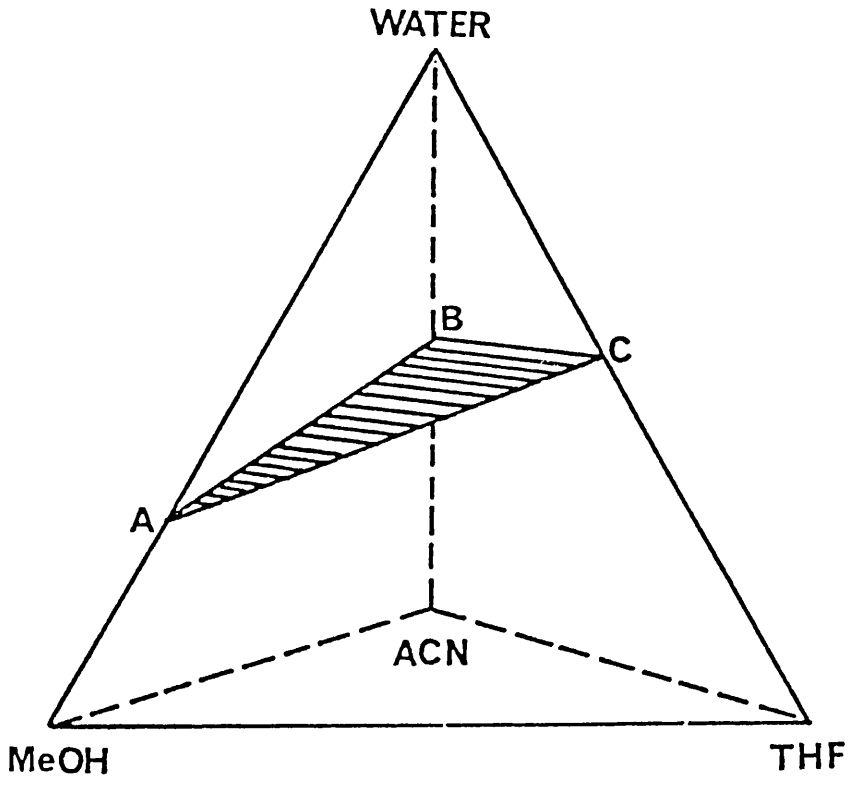


Fig.5.1 Multisolvent space showing a hypothetical iso-eluotropic plane.

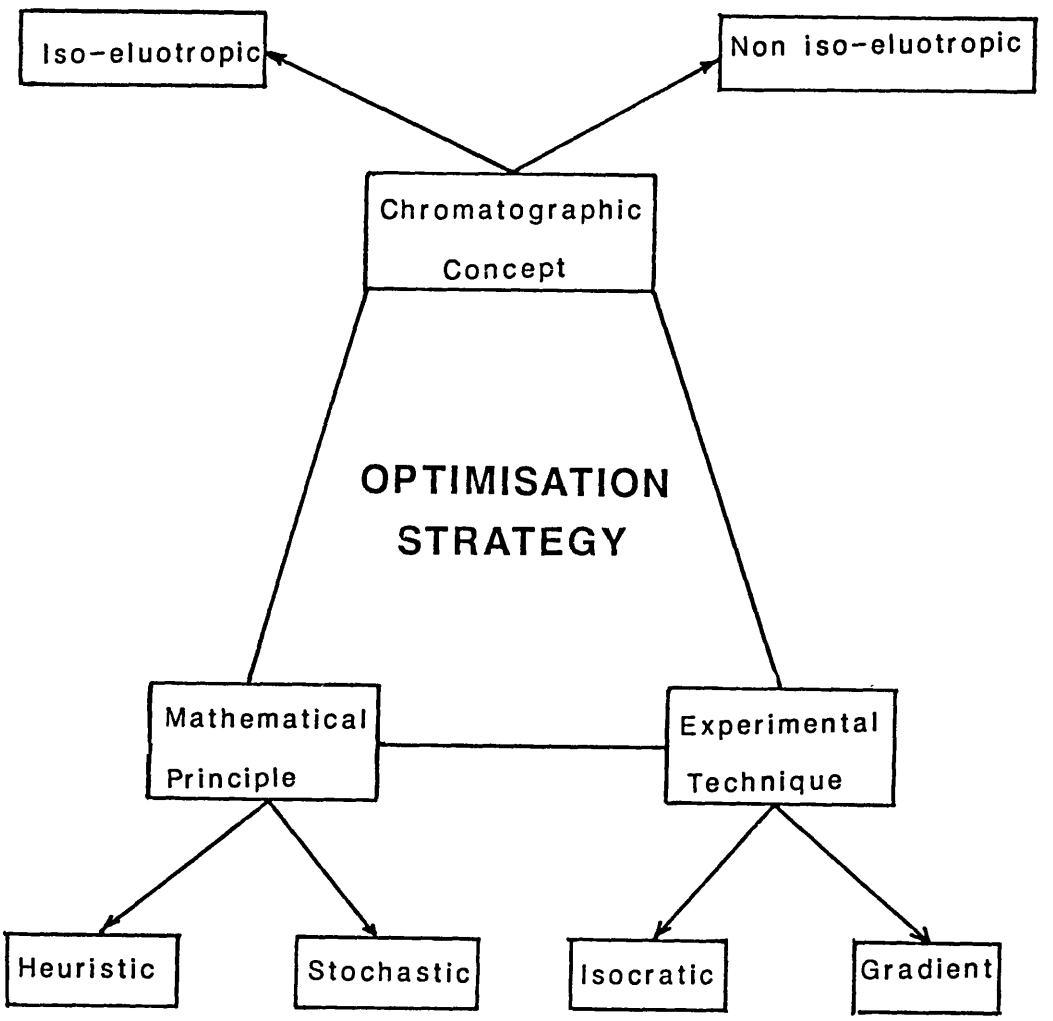
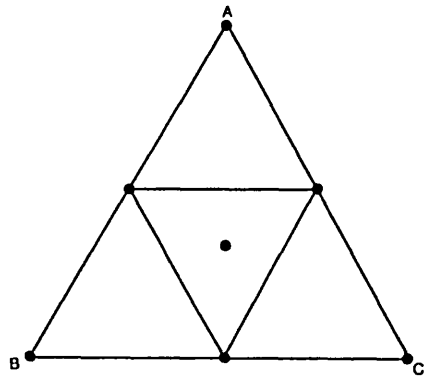
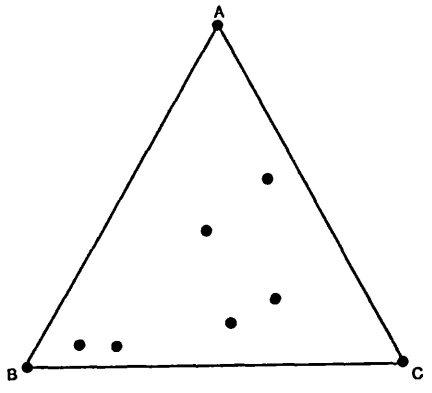


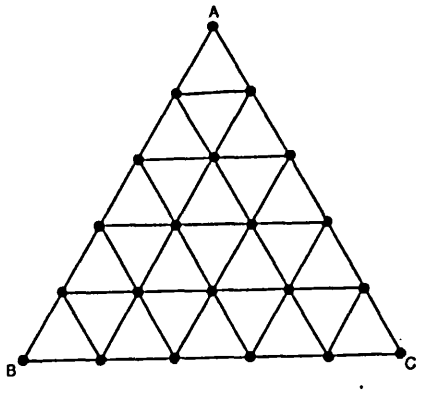
Fig.5.2 Schematic diagram showing three basic components and their sub-divisions of an optimisation strategy.



A: Fixed Design (Simplex or MDS)



B: Open Design



C: Factorial/Grid Design (2-factor, 5-level)

Fig.5.3 Different optimisation designs employed for solvent optimisatiom: (A) Fixed, (B) Open and (C) Factorial/Grid design.

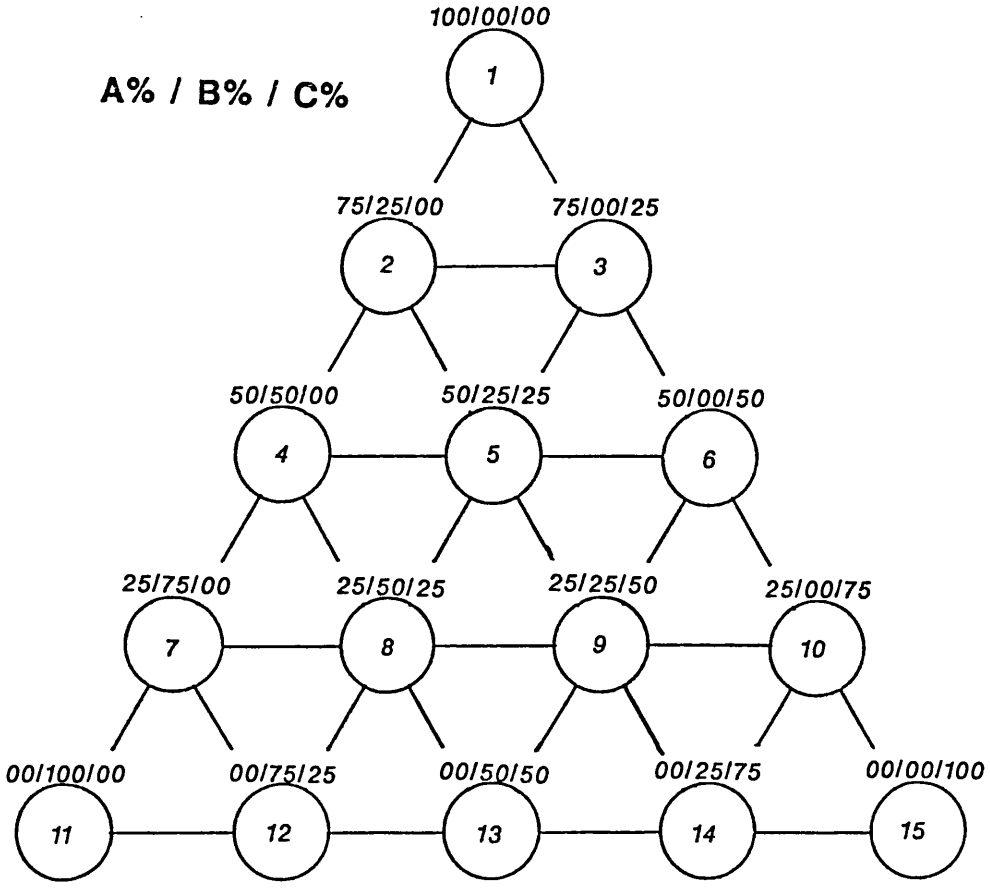


Fig.5.4 A two-factor five-level pseudo-factorial design. Numbers in the circle indicate the node number and the proportions of pseudo-solvents A/B/C are shown above each node.

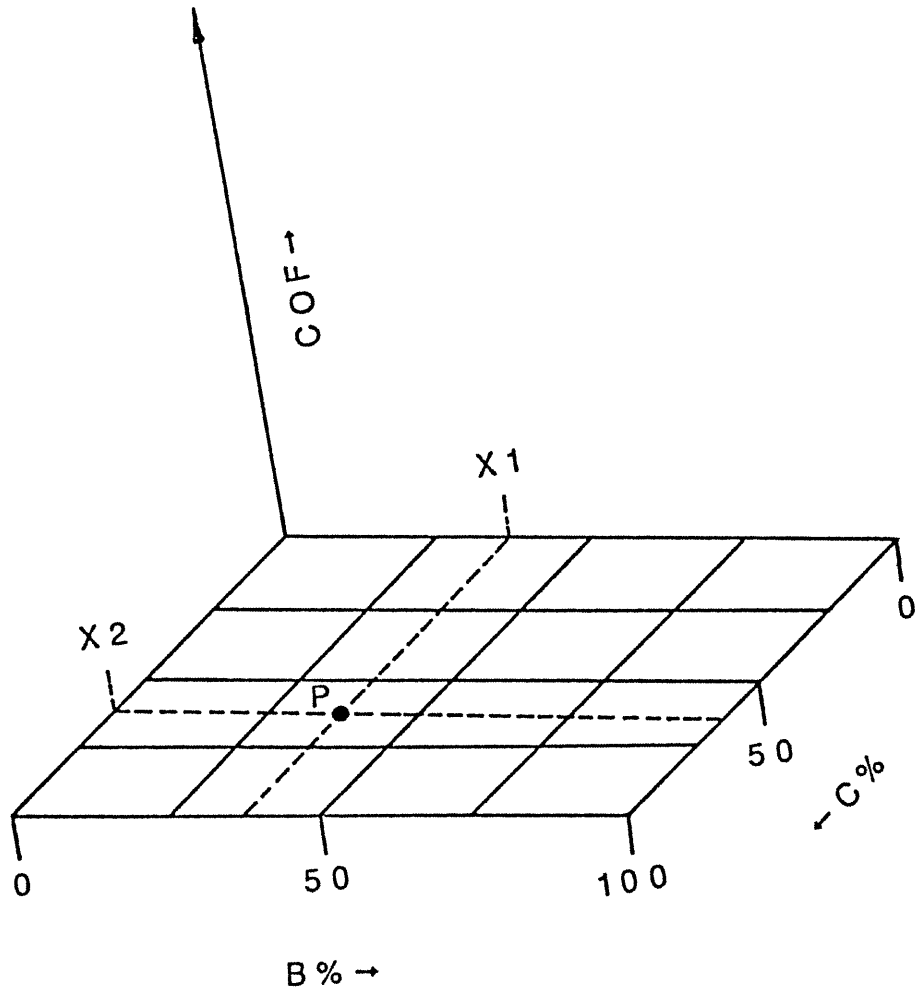


Fig.5.5 Principle of the polynomial interpolation at point 'P' from cartesian arrangement of the data.

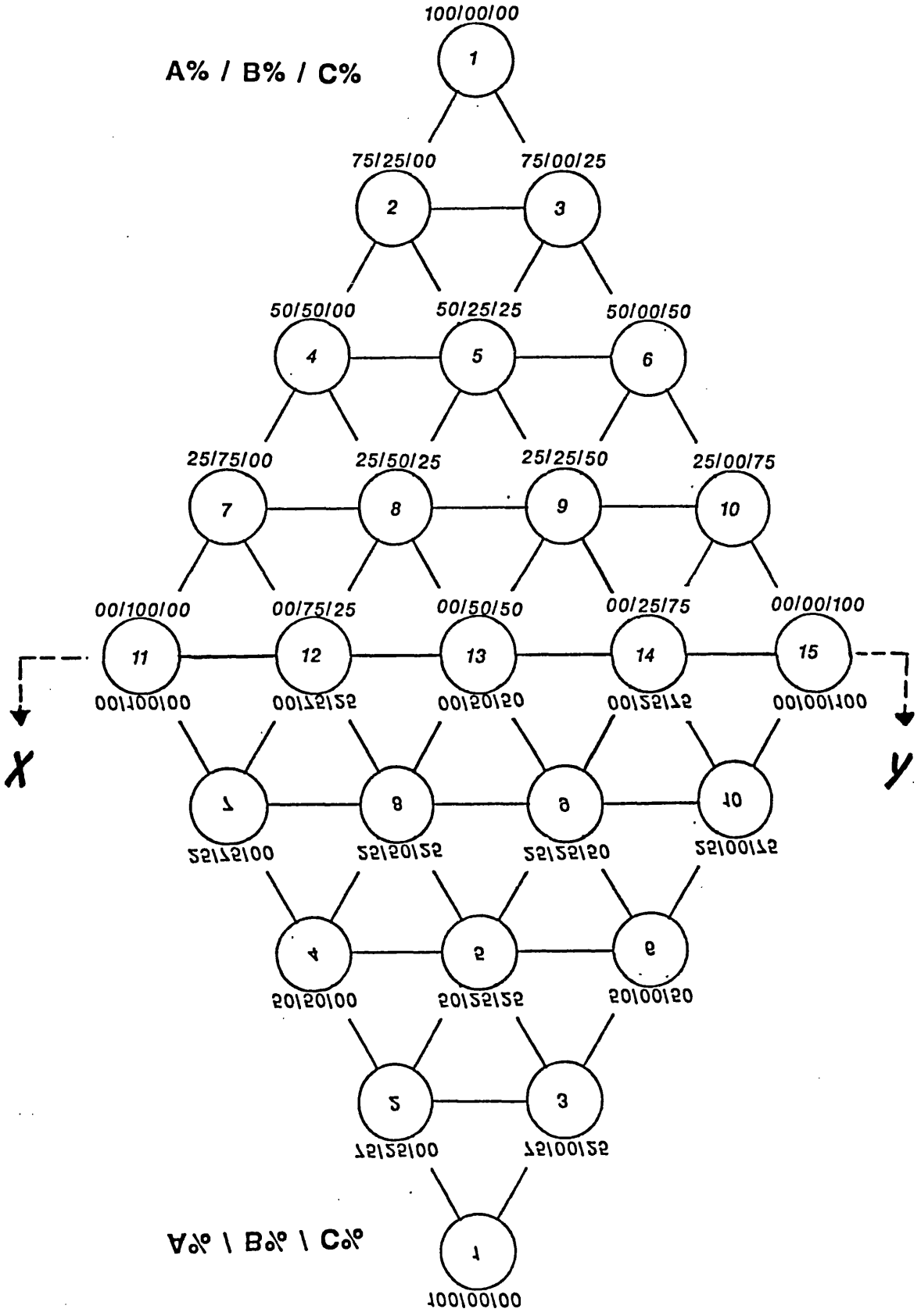


Fig.5.6 Reflectiion of the solvent-parameter space of a 2-factor 5-level design in X-Y plane.

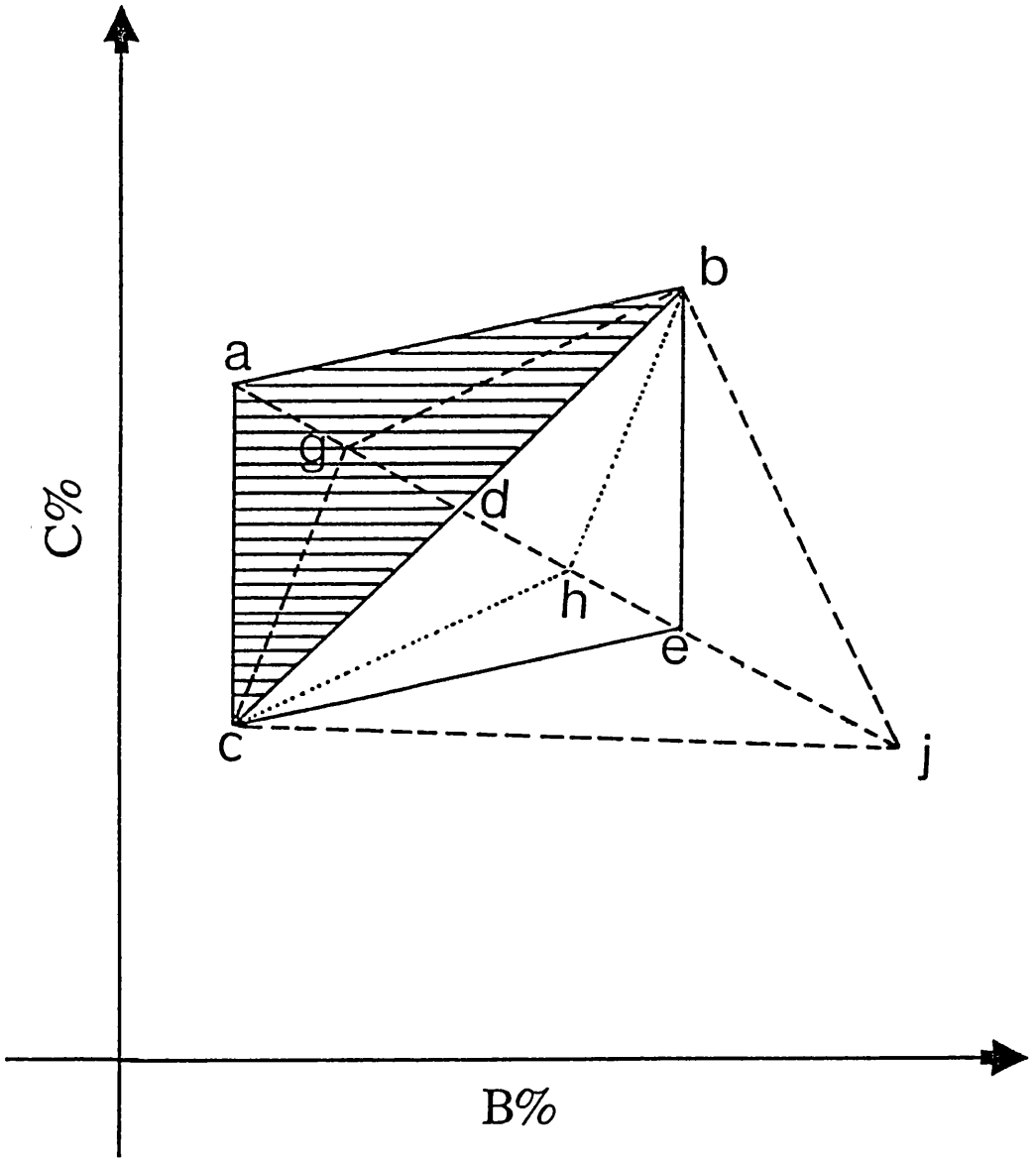


Fig.5.7 Basic principle of up-hill simplex search. Initial simplex defined by triangle abc with point 'a' corresponding a worst response as compared to point 'b' and 'c'. Possibilities for the movement of such a simplex by reflection (e), extension(j) or contraction (h,g) are shown.

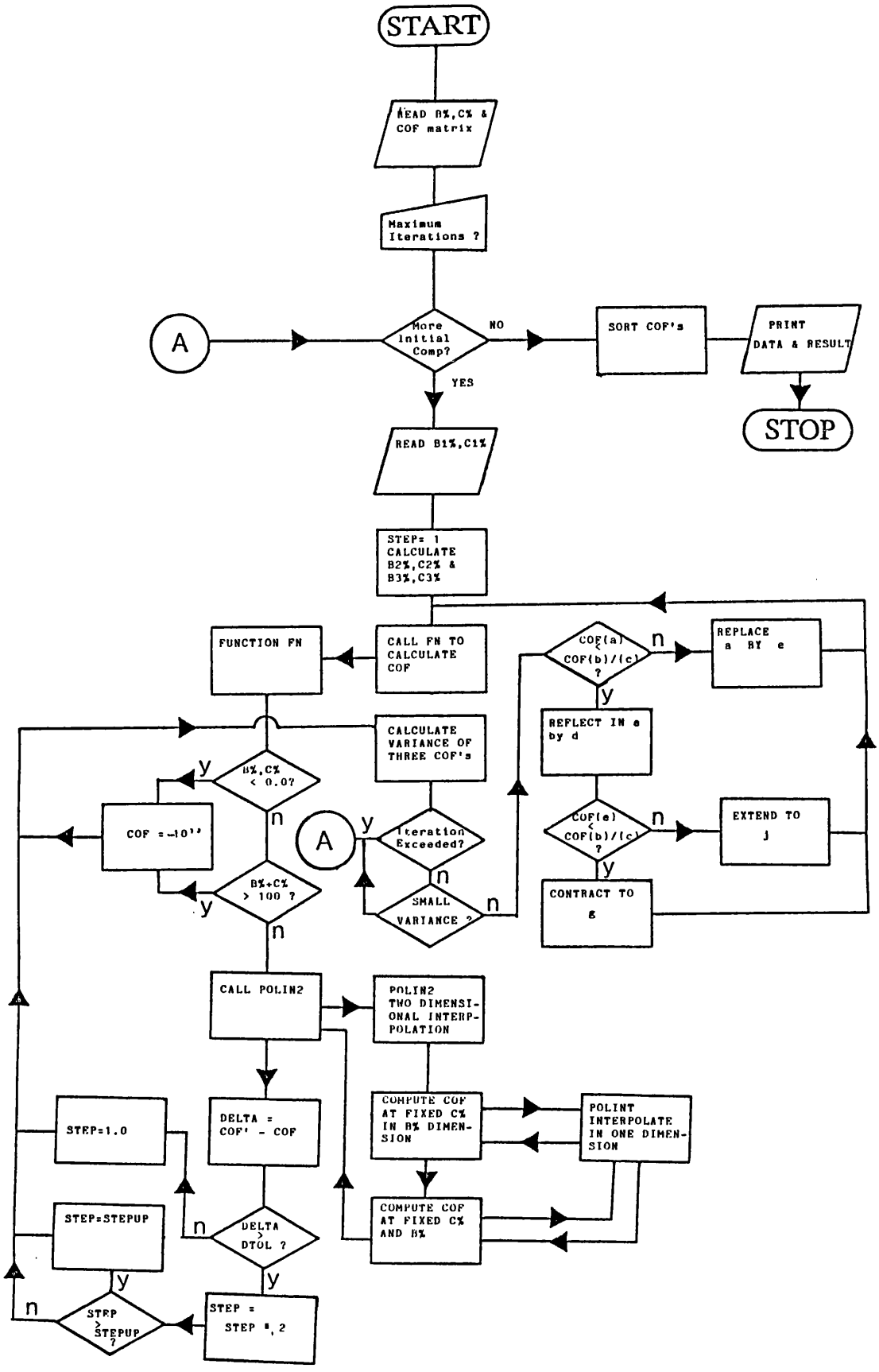


Fig.5.8

FLOW CHART SHOWING MAJOR PROCESSING AND DECISION STEPS IN ORIENT PROGRAMME

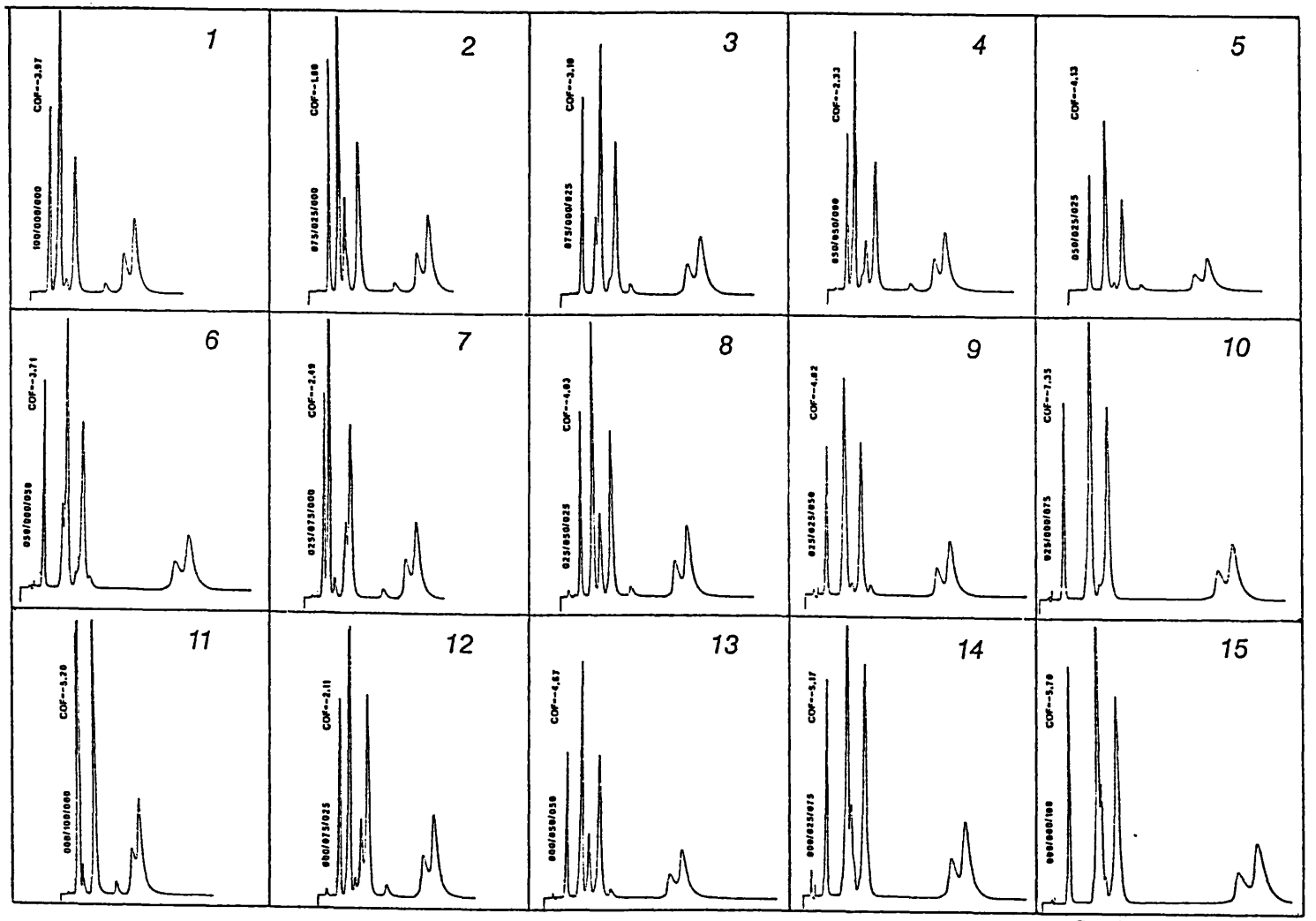
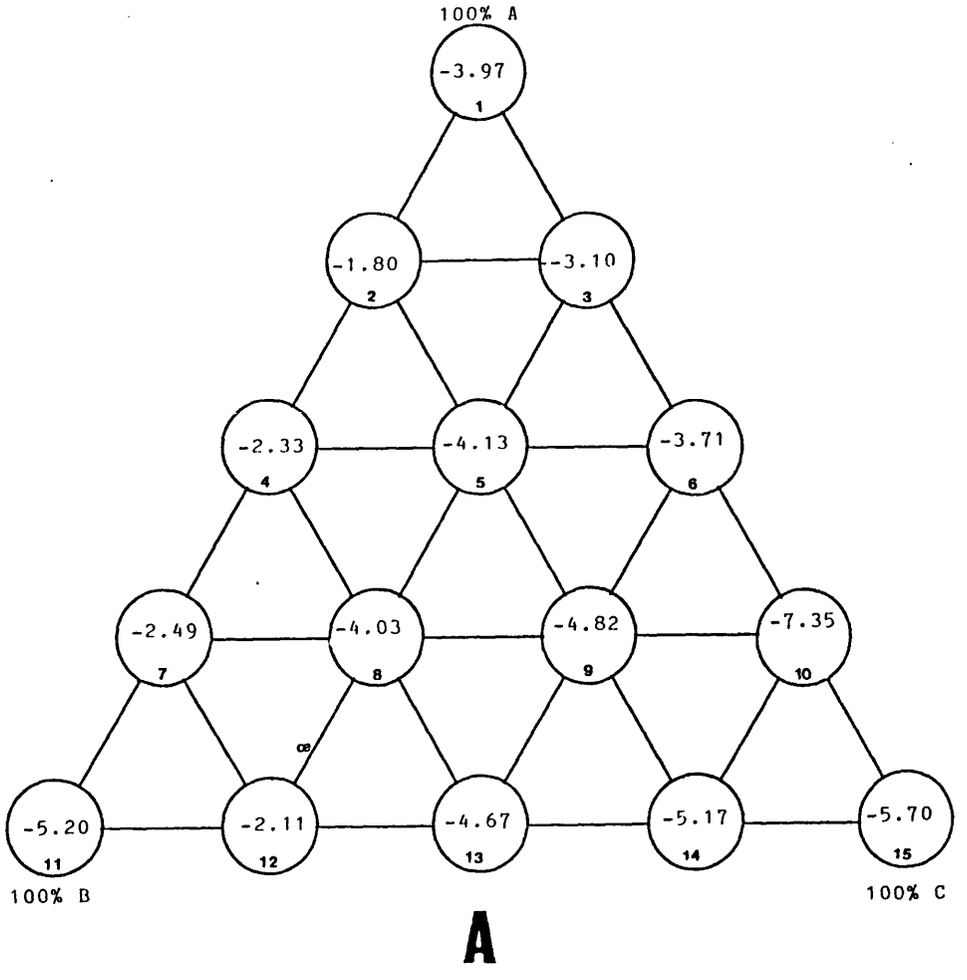


Fig.5.9 Experimental chromatograms obtained according to two factor 5-level design with the corresponding COF value, proportion of pseudo-solvents A/B/C and Node number as given in Fig.5.2.



B% \ C%	0	25	50	75	100
0	1 -3.97	3 -3.10	6 -3.71	10 -7.35	15 -5.70
25	2 -1.80	5 -4.13	9 -4.82	14 -5.17	-7.35
50	4 -2.33	8 -4.03	13 -4.67	-4.82	-3.71
75	7 -2.49	12 -2.11	-4.03	-4.13	-3.10
100	11 -5.20	-2.49	-2.33	-1.80	-3.97

B

Fig.5.10 COF values for the experimental chromatogram. (A) shows the arrangement according to 2-factor 5-level design and (B) shows the pseudo-factorial matrix obtained by taking reflection in X-Y diagonal.

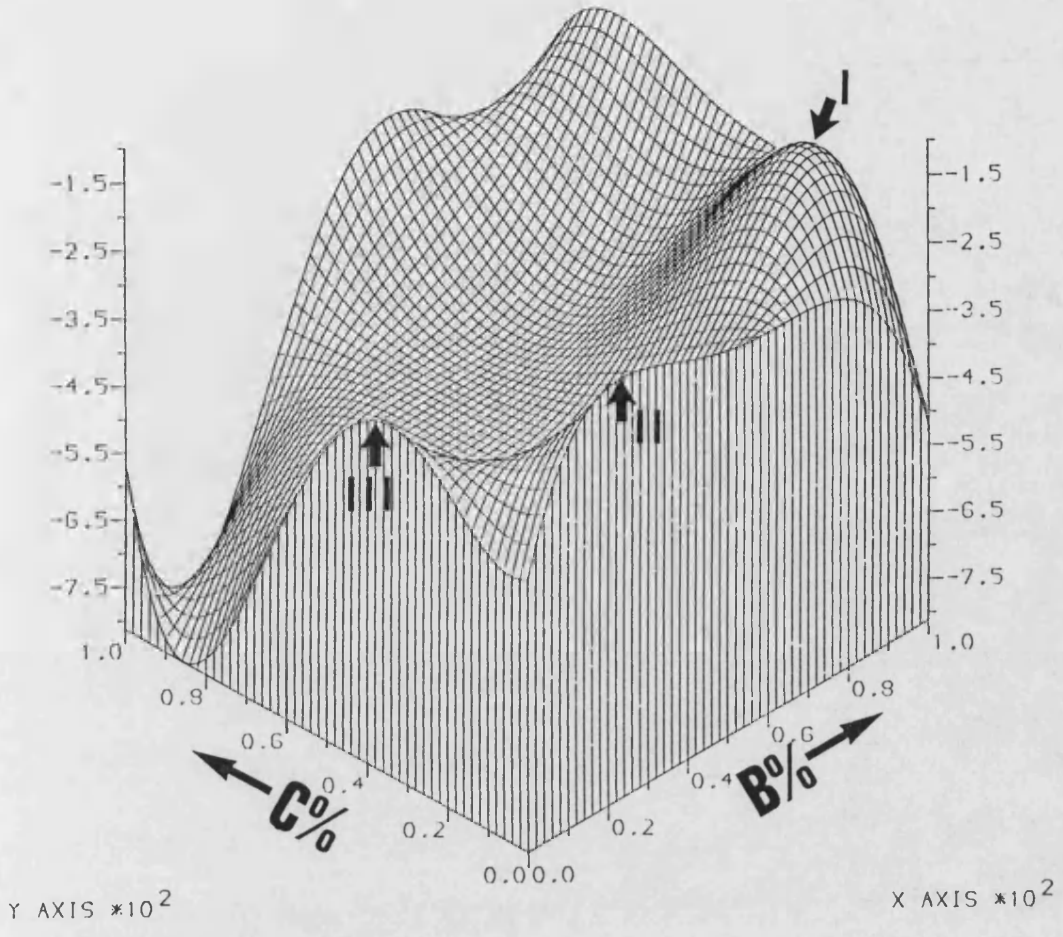


Fig.5.11 Three-dimensional view of the response surface for a two-factor five-level experimental design. Optima (I, II and III) are indicated by '->'.

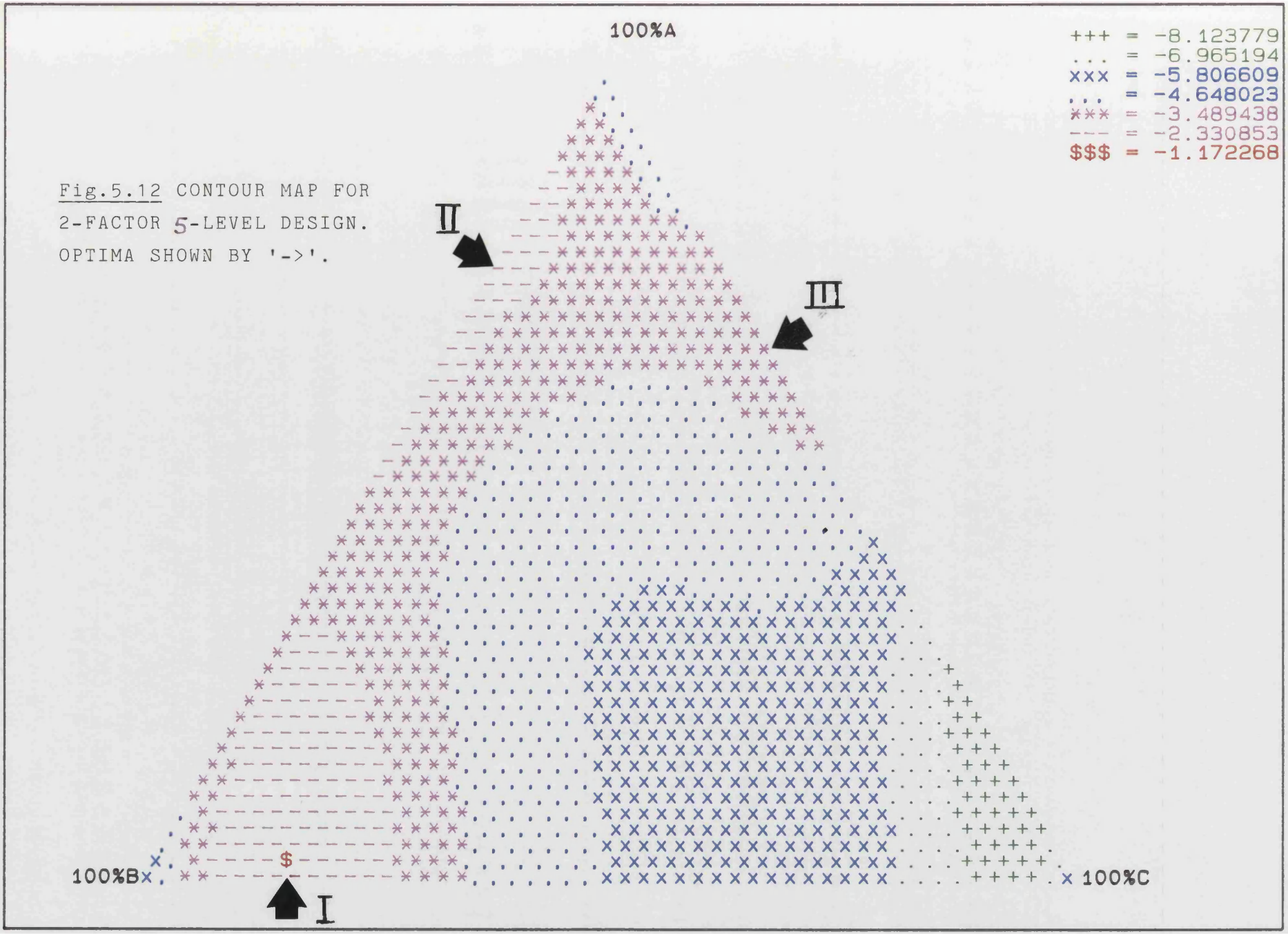


Fig.5.12 CONTOUR MAP FOR 2-FACTOR 5-LEVEL DESIGN. OPTIMA SHOWN BY '->'.

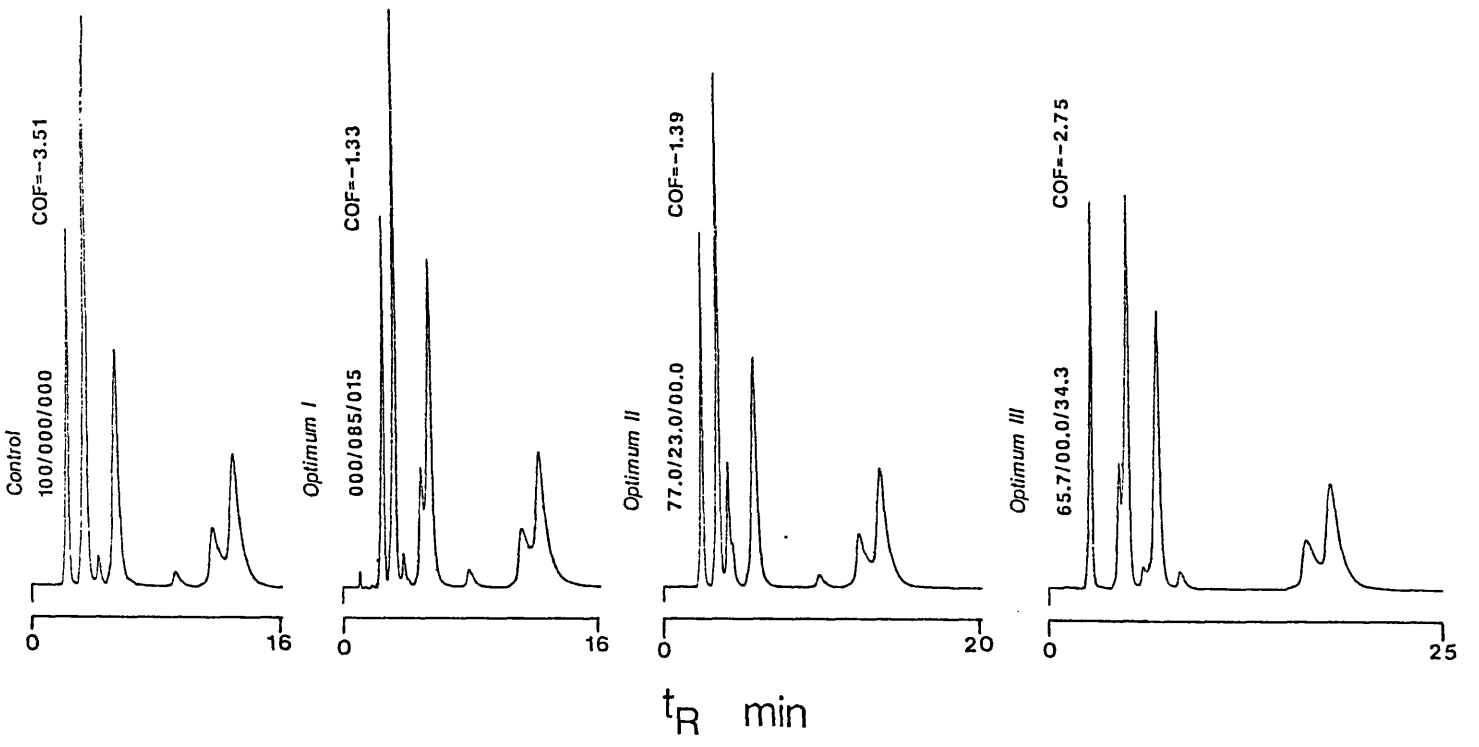


Fig.5.13 Experimental chromatograms for the optima (I, II and III) predicted by 'ORIENT' and for control.

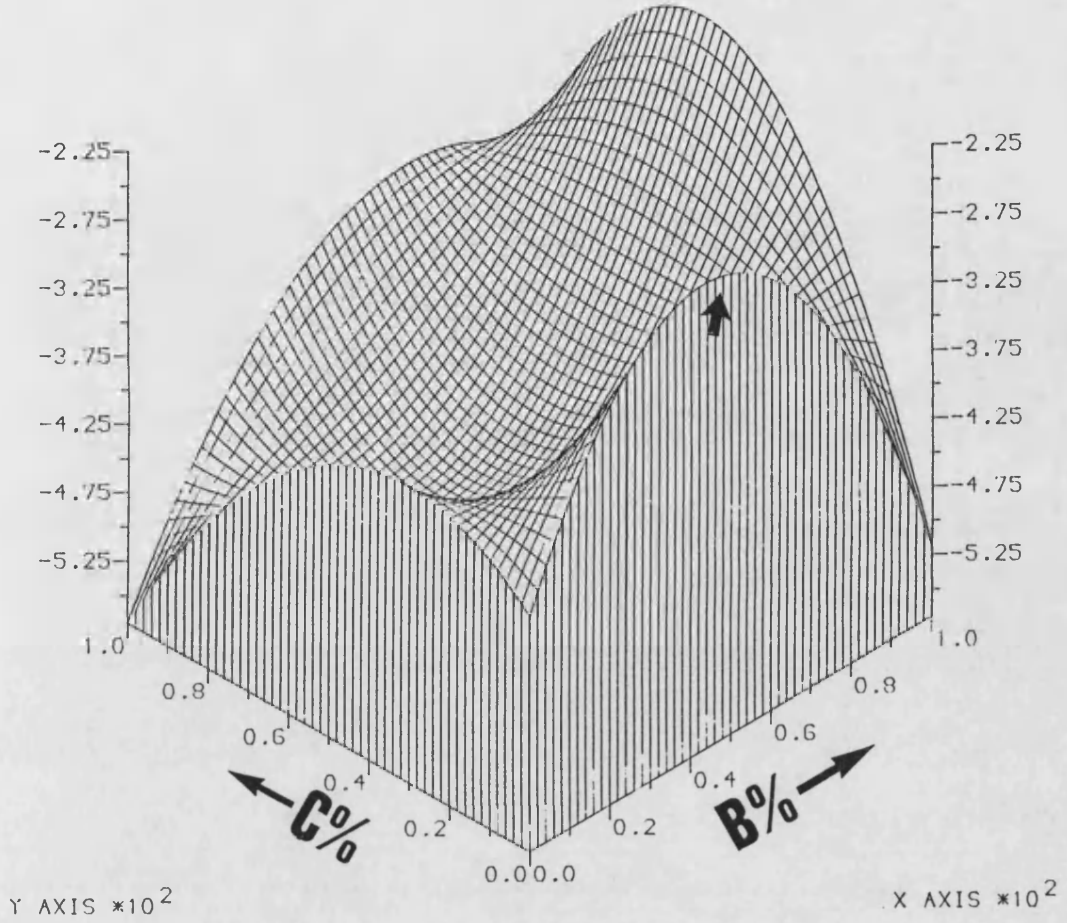


Fig.5.14 Three-dimensional view of response surface for two factor threee-level design. '->' indicates the predicted optimum.

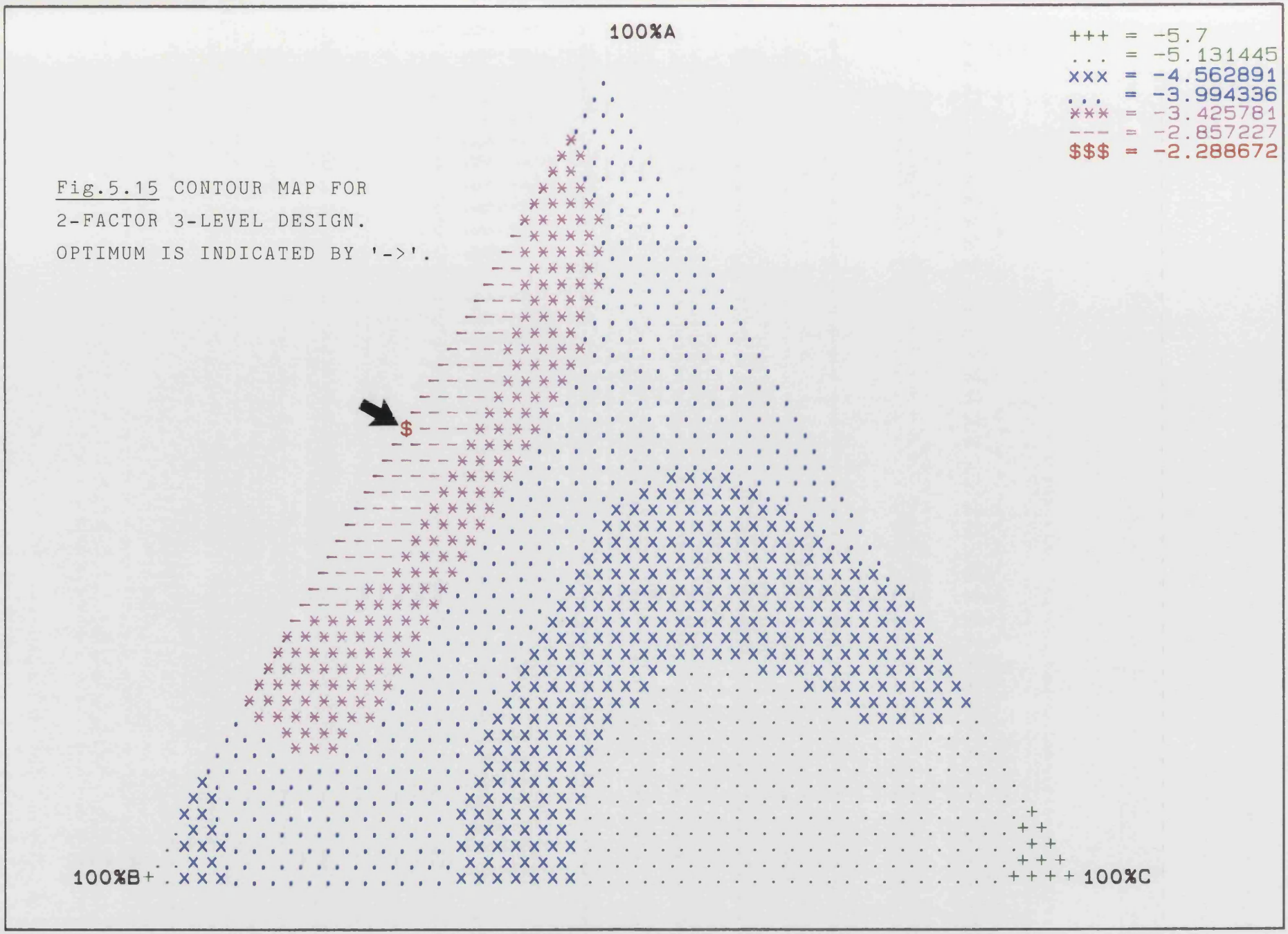


Fig.5.15 CONTOUR MAP FOR
2-FACTOR 3-LEVEL DESIGN.
OPTIMUM IS INDICATED BY '->'.

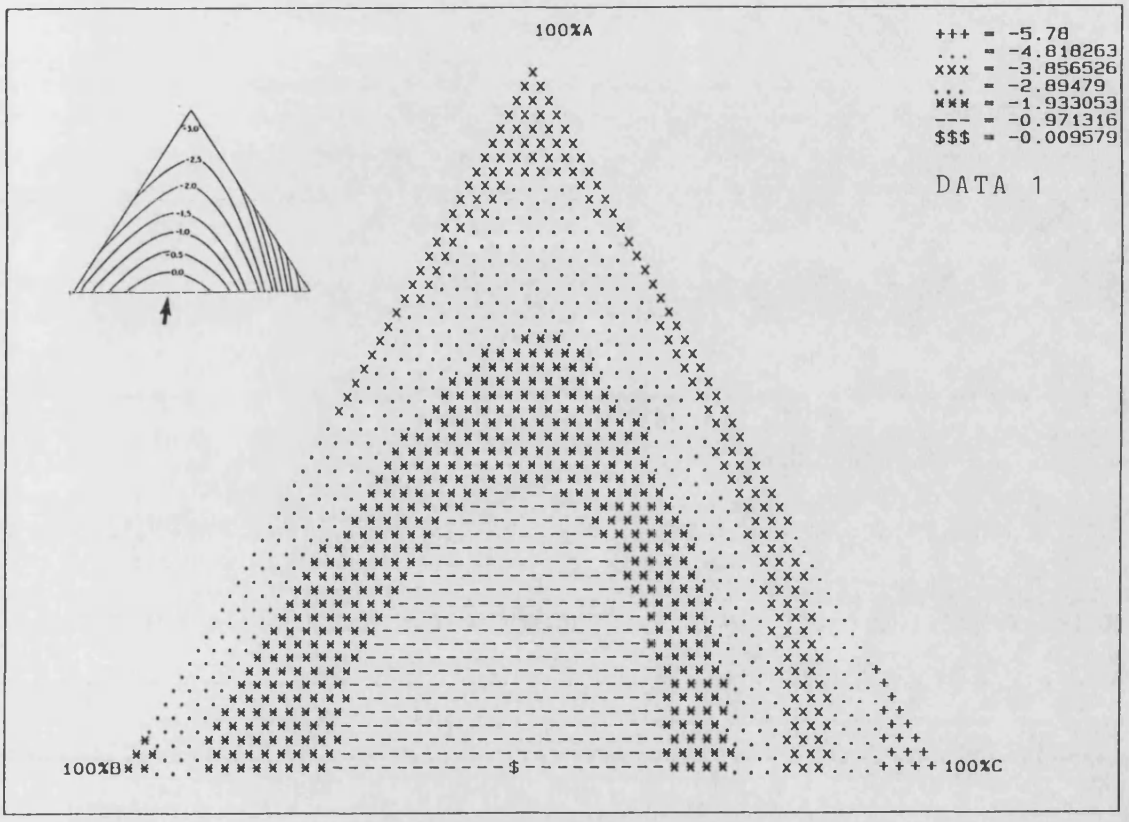


Fig.5.16 Contour plot for two factor three-level design for DATA I. The inset shows the reported map. Where '$' indicates the optimum predicted by 'ORIENT' and '->' for the optimum reported.

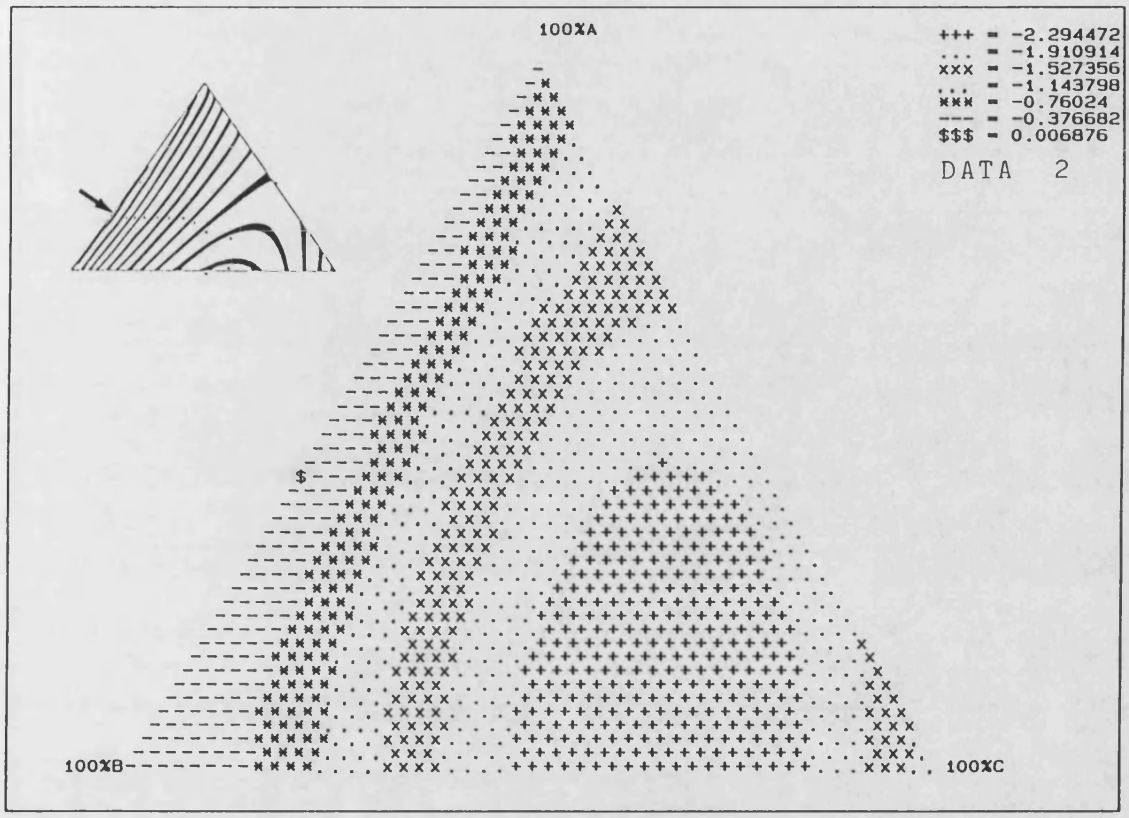


Fig.5.17 Contour plot for two factor three-level design for DATA 2. The inset shows the reported map. Where '$' indicates the optimum predicted by 'ORIENT' and '->' for the optimum reported.

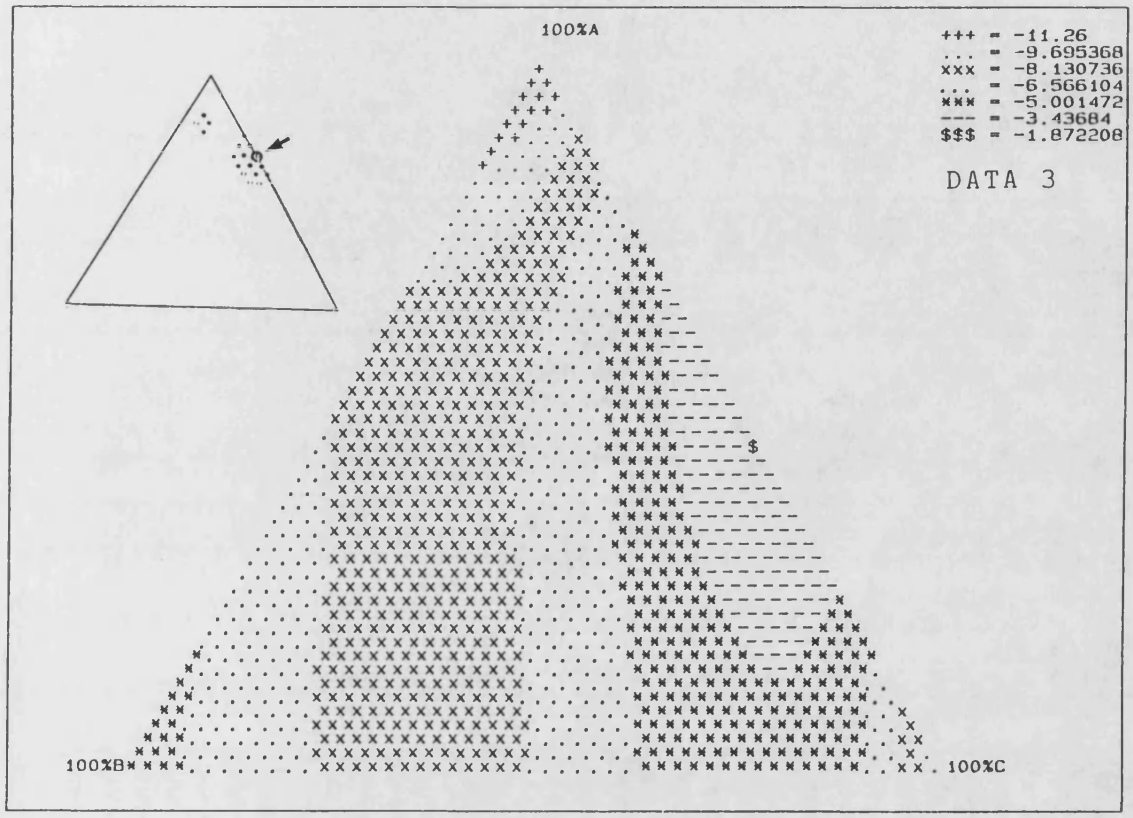
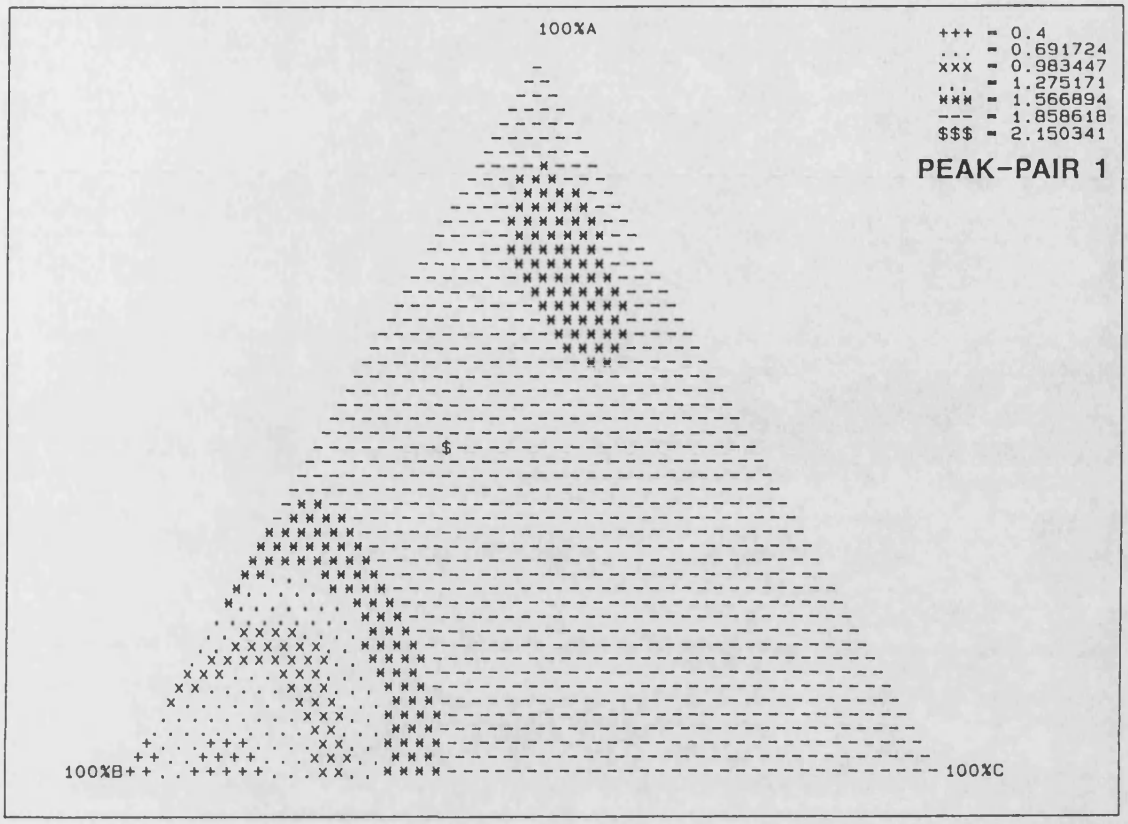


Fig.5.18 Contour plot for two factor three-level design for DATA 3. The inset shows the reported map. Where '$' indicates the optimum predicted by 'ORIENT' and '->' for the optimum reported.



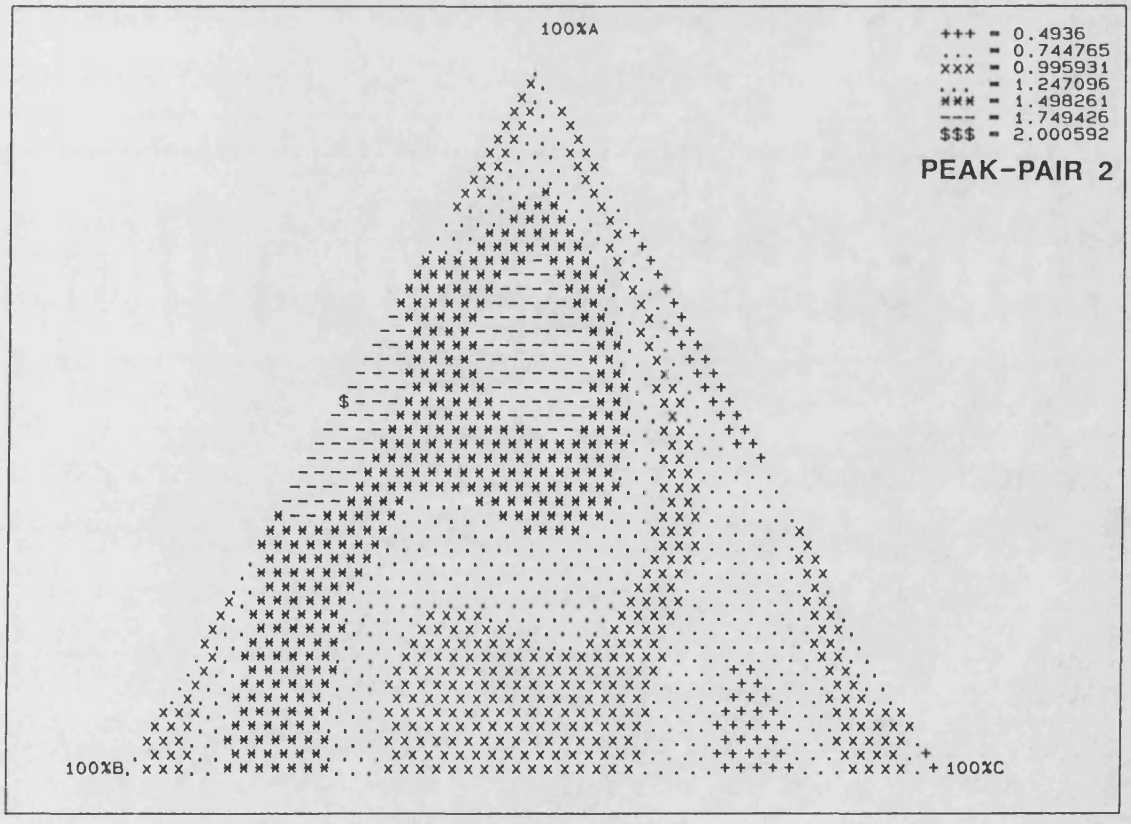
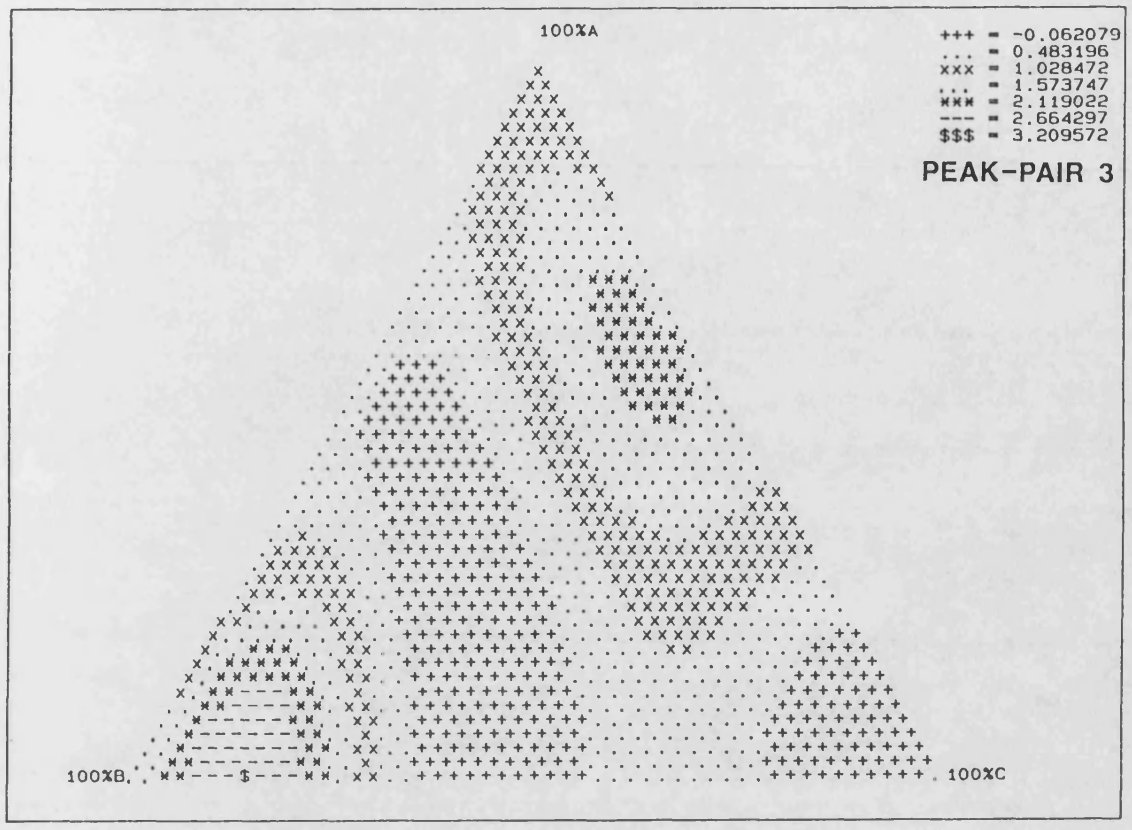


Fig.5.19(1),(2) Resolution map for peak-pair 1 and 2 from 2-factor 5-level experimental data.



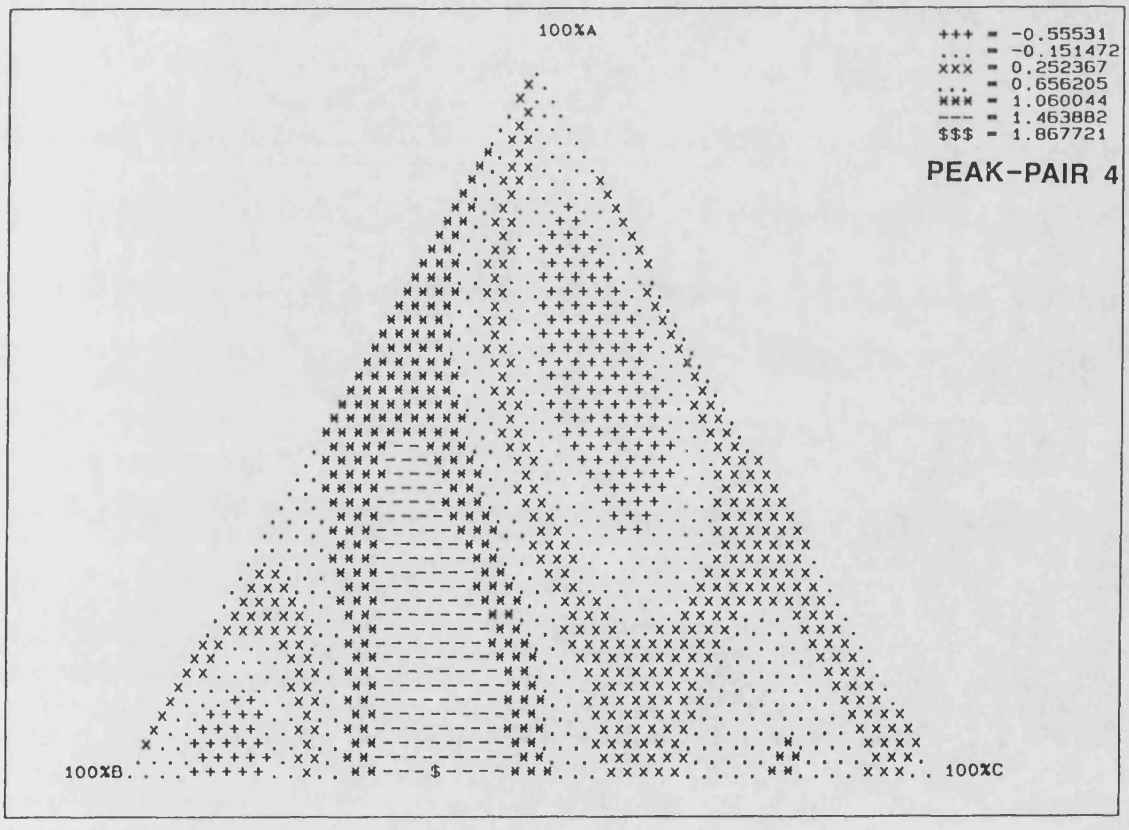
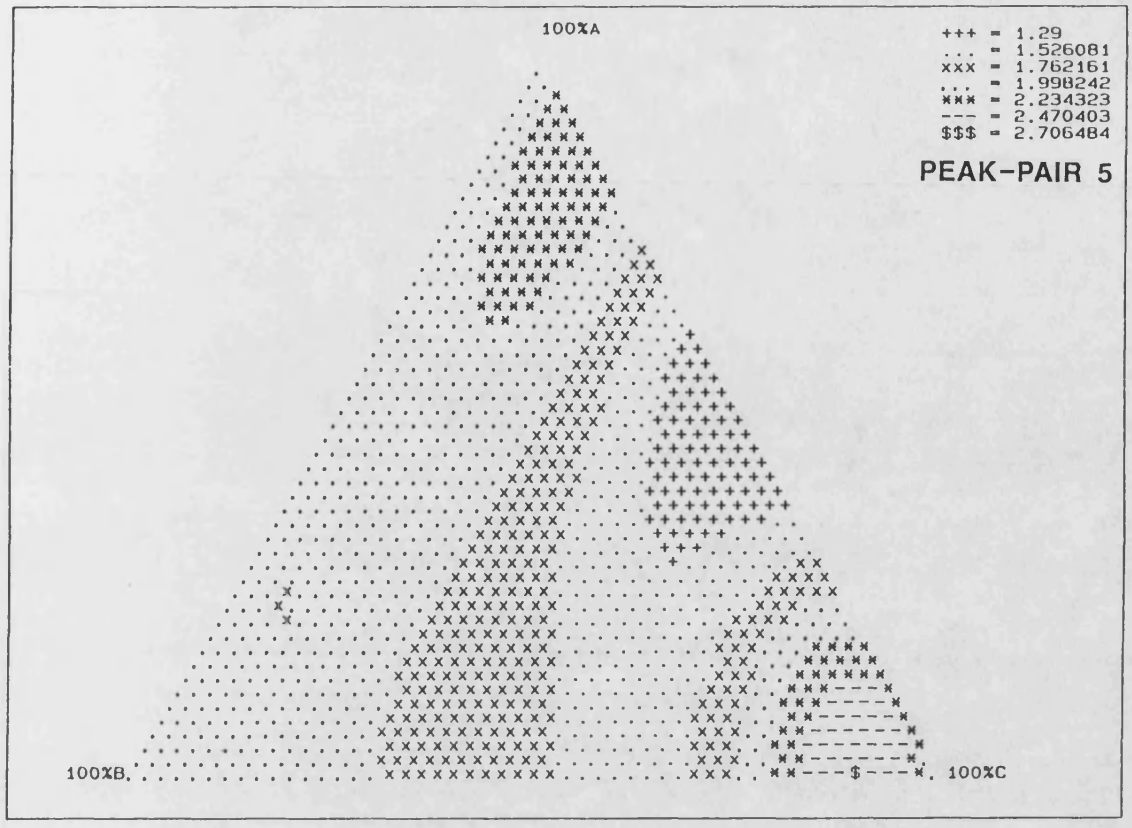


Fig.5.19(3),(4) Resolution map for peak-pair 3 and 4 from 2-factor 5-level experimental data.



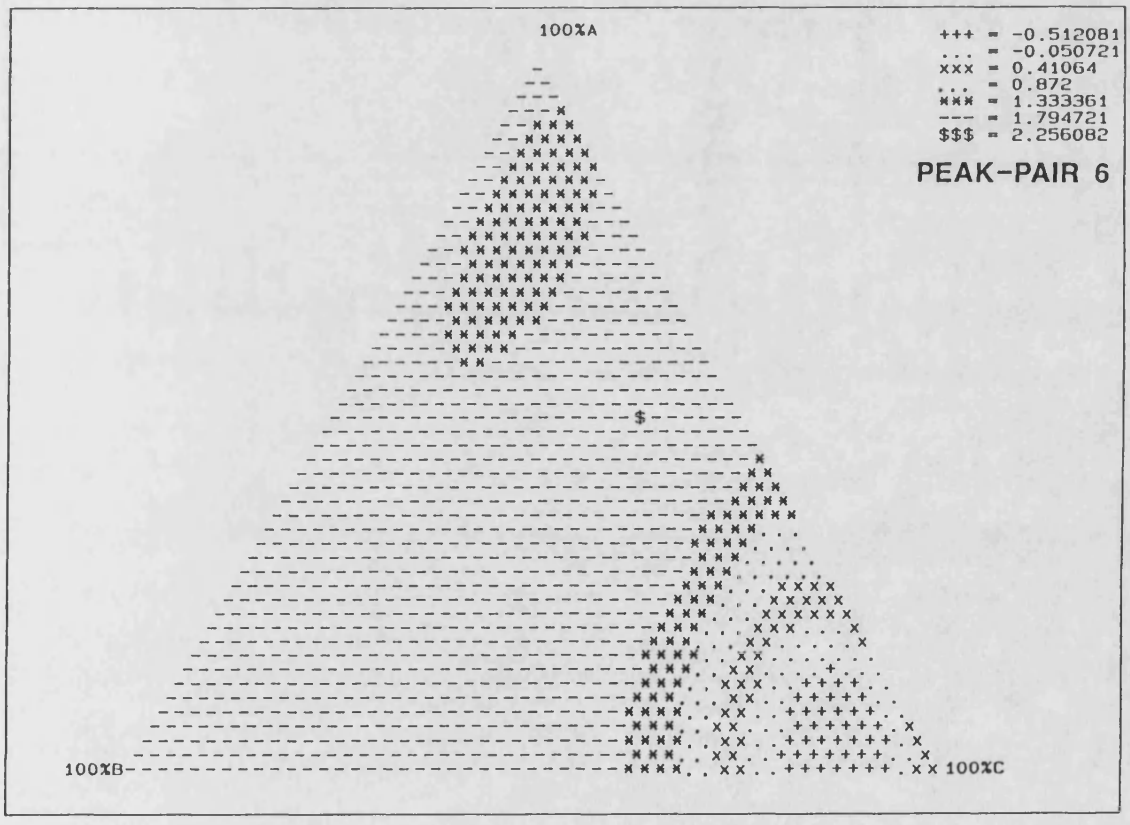


Fig.5.19(5),(6) Resolution map for peak-pair 5 and 6 from 2-factor 5-level experimental data.

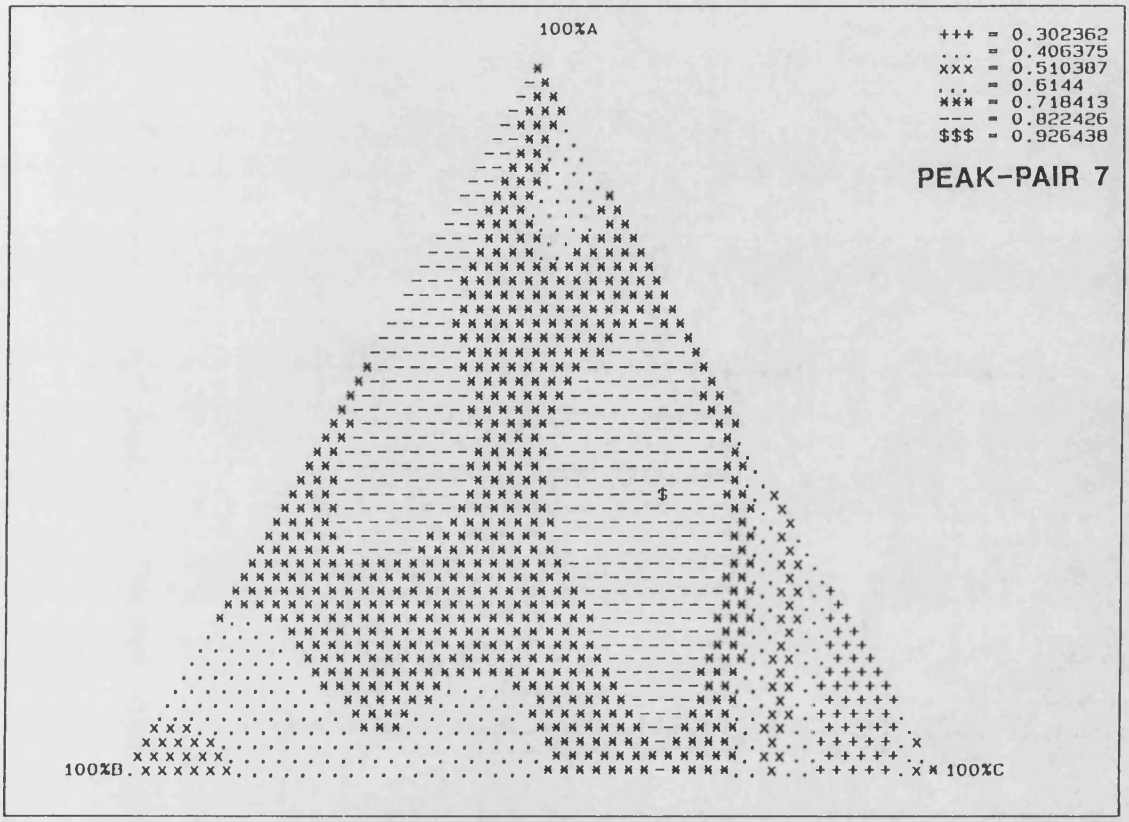


Fig.5.19(7) Resolution map for peak-pair 7 from 2-factor 5-level experimental data.

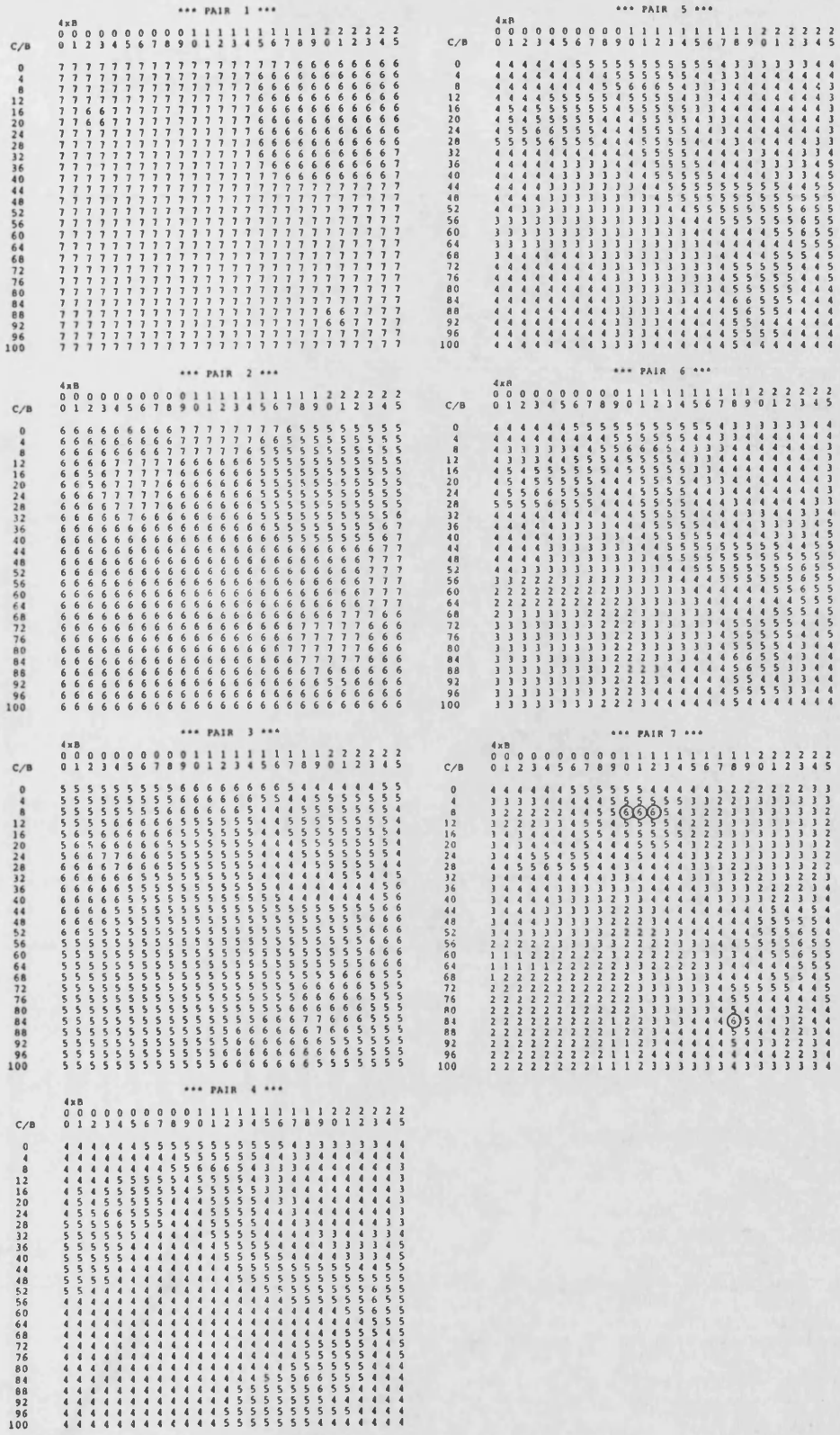


Fig.5.20 Numerograms for peak-pair 1 to 7. Optima are circled in the final (Pair 7) numerogram.

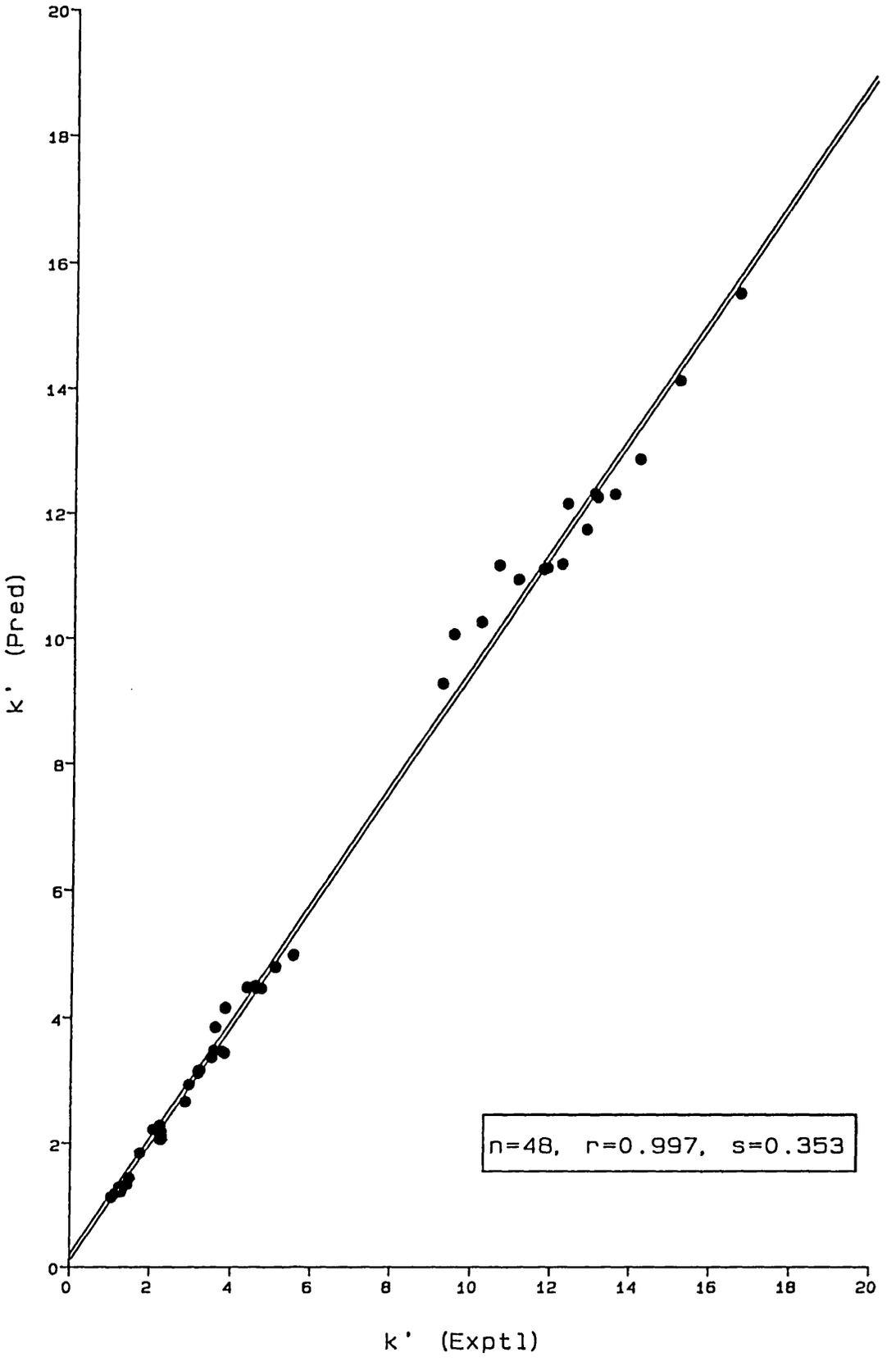

CORRELATION BETWEEN EXPERIMENTAL AND
PREDICTED RETENTIONS (k') BY "SIMULA"
k' (Pred)
k' (Exptl)
0
2
4
6
8
10
12
14
16
18
20
n=48, r=0.997, s=0.353

Fig.5.21

CORRELATION BETWEEN EXPERIMENTAL AND PREDICTED RETENTIONS (k') BY "SIMULA" FOR LITERATURE DATA

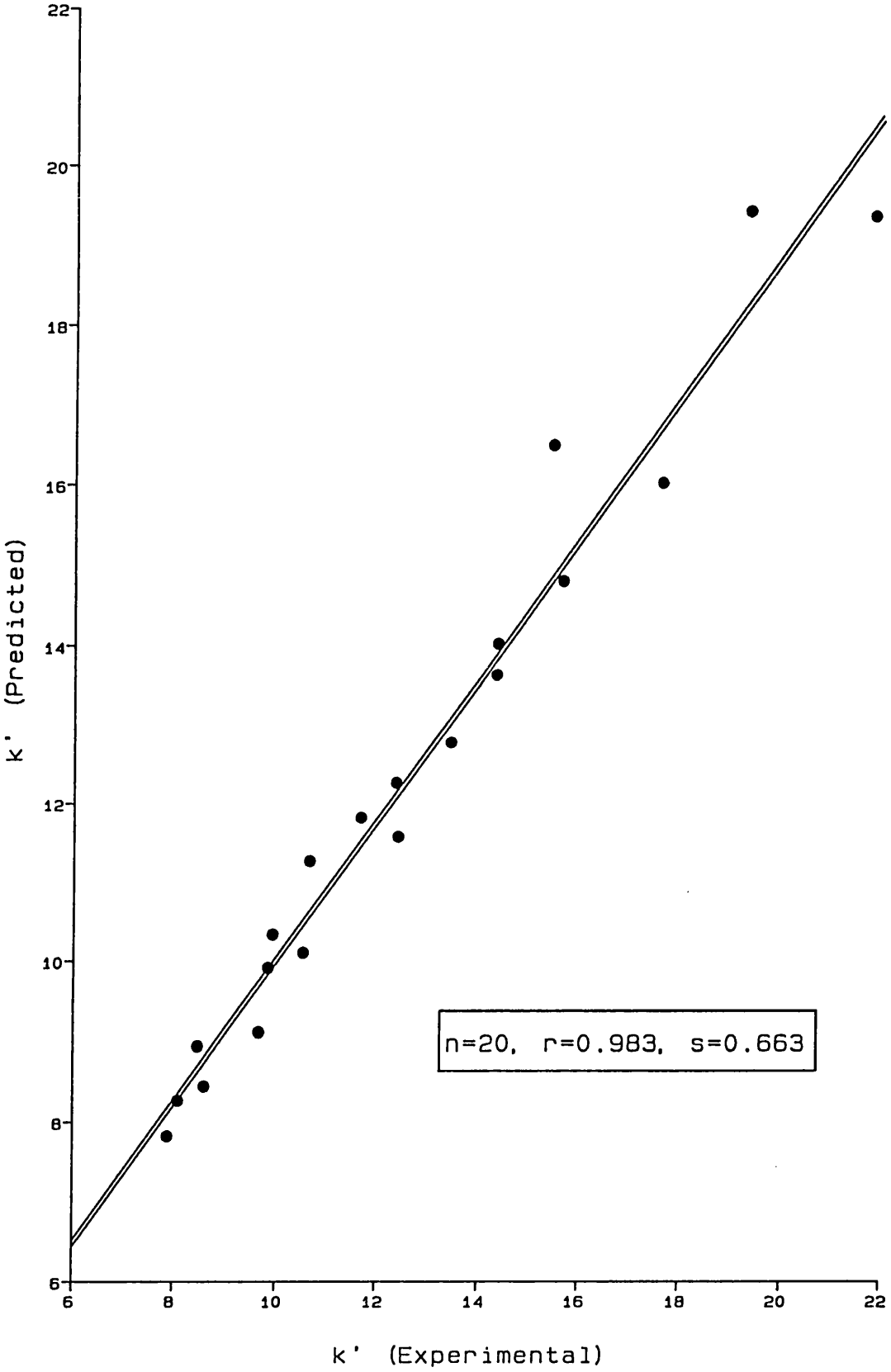


Fig.5.22

BIBLIOGRAPHY

1.1 R.E.Majors, J.Chromatogr.Sci.,18(1980)393

1.2 T.L.Hafkenscheid and E.Tomlinson, "Advances in Chromatography",Vol. 25, Marcell Dekker Inc., New York, 1985, p1

1.3 E.Tomlinson, J. Chromatogr., 113(1975)1.

1.4 G.J.Sewell, Ph.D. Thesis, "Studies on the Microbial N-dealkylation of Drug Molecules",Bath University, U.K., 1982.

1.5 M.Gibson, Ph.D. Thesis, "Analytical Studies on the N-dealkylation of Drog Molecules by Cunninghamella sp.", Bath University, U.K., 1984.

1.6 L.R.Snyder J.Chromatogr.Sci., 16(1978)223

1.7 L.R.Snyder, J.W.Dolan and J.R.Gant, J. Chromatogr., 165(1979)3

1.8 R.Tijssen, H.A.H.Billiet and P.J.Schoenmakers, J. Chromatogr., 122(1976)185

1.9 P.J.Schoenmakers, H.A.H.Billiet and L.de Galan, J. Chromatogr., 205(1981)13.

1.10 P.J.Schoenmakers, H.A.H.Billiet, and L.de Galan, J.Chromatogr., 185(1979)175.

1.11 S.Sekulic, P.R.Haddad and C.J.Lamberton, J. Chromatogr., 363(1986)125.

1.12 J.C.Berridge, J.Chromatogr., 244(1982)1.

1.13 J.C.Berridge, Chromatographia, 16(1982)172.

1.14 R.J.Laub and J.H.Purnell, J. Chromatogr., 112(1975)71.

1.15 P.J.Schoenmakers, "Optimisation of Chromatographic Selectivity, aguide to method delvelopment", J. Chromatogr. Library, Vol.35., Elsevier Sci. Pub., Amsterdam, 1986, Chapter 4.

2.1 R.V.Smith and J.P.Rosazza, Arch. Biochem. Biophy., 161(1974)551.

2.2 J.B.Jones, C.J.Sih and D.Perlman (Ed), "Application of Biochemical Systems in Organic Chemistry", J.Wiley Sons, New York, 1967, p495.

2.3 K.Kieslich, Biotechnol. Lett., 2(1980)211.

2.4 W.Charney and H.L.Herzog, "Microbial Transformation of Steroids", Academic Press, New York, 1967.

2.5 H.Iizuka and A.Naito, "Microbila Transformation of Steroids and Alkaloids", University Park Press, State College, Pa., 1967.

2.6 G.Fonken and R.S.Johnson, "Chemical Oxidation with Microorganisms", Dekker, New York, 1972.

2.7 L.L.Wallen, F.H.Stodola and R.W.Jackson, "Type Reaction in Fermentation Chemistry", Bull. ARS-71-13, Agricultural Research Service, U.S. Department of Agriculture, Washington, D.C., 1959.

2.8 C.Tamm, Angew. Chem. Int. Ed, 1(1962)178.

2.9 D.Pearlman and O.K.Sebek, Pure. Appl. Microbiol., 26(1973)98.

2.10 O.K.Sebek, Lloydia, 37(1974)115.

2.11 G.J.Sewell, Ph.D. Thesis, "Studies on the Microbial N-dealkylation of Drug Molecules",Bath University, U.K., 1982.

2.12 M.Gibson, Ph.D. Thesis, "Analytical Studies on the N-dealkylation of Drog Molecules by Cunninghamella sp.", Bath University, U.K., 1984.

2.13 T.L.Hafkenscheid and E.Tomlinson, "Advances in Chromatography",Vol. 25, Marcell Dekker Inc., New York, 1985, p1

2.14 C.Horvath, W.Melander, I.Molnar, J.Chromatogr., 125(1976)129.

2.15 Ref.2.13 Table 1 contains a comprehensive list of related publication.

2.16 R.F.Rekker, "The Hydrophobic Fragmental Constant", Elsevier, Amsterdam, 1977.

2.17 L.R.Snyder J.Chromatogr.Sci., 16(1978)223

2.18 J.W.Bridges and L.F.Chesseaud (Ed), "Prograss in Drug Metabolism", Vol.8, Taylor anf Francis, London, 1984.

3.1 R.E.Majors, J.Chromatogr.Sci.,18(1980)393

3.2 J.L.Glajch, J.J.Kirkland, K.M.Squire and J.M.Minor, J.Chromatogr., 199(1980)57

3.3 P.J.Schoenmakers, H.A.H.Billiet and L.de Galan, J. Chromatogr., 205:(1981)13

3.4 L.R.Snyder J.Chromatogr.Sci., 16(1978)223

3.5 L.R.Snyder, J.W.Dolan and J.R.Gant, J. Chromatogr., 165(1979)3

3.6 R.Tijssen, H.A.H.Billiet and P.J.Schoenmakers, J. Chromatogr., 122(1976)185

3.7 L.R.Snyder (Ed.) "Principles of adsorption chromatography", Marcell-Dekker Inc., New York, 1968, pp194-195.

3.8 H.Colin, C.Eon and G.Guiochon, J. Chromatogr., 122(1976)223

3.9 C.Horvath, W.Melander, I.Molnar, J.Chromatogr., 125(1976)129.

3.10 B.L.Karger, J.R.Gant, A.Hortkopf and P.Weiner, J. Chromatogr., 128(1976)65

3.11 N.Tanaka and E.R.Thornton, J.Am.Chem.Soc., 99(1977)7300

3.12 D.C.Locke, J.Chromatogr., 12(1974)433

3.13 C.Horvath and W.Melander, J.Chromatogr.Sci., 15(1977)393

3.14 W.R.Melander and C.Horvath, Chromatographia, 18(1984)353

3.15 W.R.Melander and C.Horvath, "High Performance Liquid Chromatography", Vol 2.,Academic Press, New York, 1980,p 113

3.16 R.E.Majors, in C.Horvath (Editor) "High Performance Liquid Chromatography" Vol 1, Academic Press, New York, 1980, p75.

3.17 J.Oscik, Przem. Chem., 44(1965)129

3.18 T.L.Hafkenscheid and E.Tomlinson, "Advances in Chromatography",Vol. 25, Marcell Dekker Inc., New York, 1985, p1

3.19 L.R.Snyder J.Chromatogr.Sci., 16(1978)223

3.20 L.R.Snyder and J.J.Kirkland, "Introduction to Modern Liquid Chromatography", 2ed, Wiley-Interscience, New York, 1979

3.21 S.R.Bakalyar, R.McIlwrick and E.Roggendorf, J. Chromatogr., 142(1977)353

3.22 G.d'Agostino, L.Castagnetta, F.Mitchell and M.J.O'Hare, J.Chromatogr., 338(1985)1

3.23 G.d'Agostino, L.Castagnetta, F.Mitchell and M.J.O'Hare, J.Chromatogr., 305(1984)13

3.24 H.K.Smith, W.L.Switzer, G.W.Martin, S.A.Benezra, W.P.Wilson and D.W.Dixson, J.Chromatogr. Sci., 24(1986)70

3.25 A.C.J.H.Drouen, H.A.H.Billiet, P.J.Schoenmakers and L. de Galan, Chromatographia, 16(1982)48

3.26 L.de Galan and H.A.H.Billiet, "Advances in Chromatography", Vol.25, Marcell Dekker Inc., New York, 1985, p96

3.27 A.C.J.H.Drouen, H.A.H.Billiet and L.de Galan, J. Chromatogr., 352(1986)127

3.28 J.W.Dolan, J.R.Gant and L.R.Snyder, J. Chromatogr., 165(1979)31

3.29 A.Poile, Perkin-Elmer Anal. News, 10(1977)8

3.30 H.Englehardt, Z. Anal. Chem., 277(1975)267

3.31 P.Jandera, J.Chromatogr., 314(1984)13

3.32 P.Jandera, J.Chromatogr., 352(1986)91

3.33 P.J.Schoenmakers, H.A.H.Billiet, R.Tijssen and L.de Galan, J.Chromatogr., 149(1978)519

3.34 A.Purkayastha and J.Walkley, Can. J. Chem., 50(1972)834

3.35 A.Leo, C.Hansch and D.Elkins, Chem. Rev., 71(1971)525

3.36 S.H.Yalkowaski, A.A.Sinkula and S.C.Valvani, "Physical Chemical Properties of Drugs", Medicinal Research Series, Vol. 10, Marcell Dekker Inc., New York, 1980

3.37 C.Hansch and A.Leo, "Substituent Constants for Correlation Analysis in Chemistry and Biology", Wiley-Interscience, New York, 1979

3.38 C.F.Gerald, "Applied Numerical Analysis", Addison-Wesley, Reading, Mass., 1978

3.39 Handbook of Chemistry and Physics, CRC press, London, 1986, 65th Ed.

4.1 P.J.Schoenmakers, H.A.H.Billiet and L. de Galan, J.Chromatogr., 205(1981)13 .

4.2 S.Sekulic, P.R.Haddad and C.J.Lamberton, J. Chromatogr., 363(1986)125.

4.3 M.W.Watson and P.W.Carr, Anal.Chem., 51(1979)1835.

4.4 J.C.Berridge, J.Chromatogr., 244(1982)1.

4.5 W.Melander and Cs.Horvath, Chromatographia, 15(1982)86.

4.6 T.L.Hafkenscheid, Ph.D. Thesis, University of Amsterdam, 1983.

4.7 K.Jinno and K.Kawasaki J.Chromatogr., 298(1984)322.

4.8 K.Jinno and K.Kawasaki, Chromatographia, 17(1983)357.

4.9 K.Jinno and K.Kawasaki, Chromatographia, 17(1983)445.

4.10 K.Jinno and K.Kawasaki, Chromatographia, 18(1984)90.

4.11 T.L.Hafkenscheid and E.Tomlinson, Adv.Chromatogr., 25(1985)1.

4.12 Cs.Horvath, W.Melander and I.J.Molnar,J. Chromatogr., 125(1976)129.

4.13 C.Tanford, "The Hydrophobic Effect", Wiley-Interscience, New York, 1973.

4.14 O.Sinanoglu, in Pullman(Ed.), "Molecular Associations in Biology", Academic Press, New York, 1968, p427.

4.15 O.Sinanoglu and S.Abdulnur, Fed. Proc., 24(2)(1965)12.

4.16 N.Tanaka and E.R.Thornton, J. Am. Chem. Soc., 99(1977)7300.

4.17 B.L.Karger, J.R.Gant, A.Hartkopf and P.Weiner, J.Chromatogr., 128(1978)519.

4.18 P.J.Schoenmakers, H.A.H.Billiet, R.Tijssen, and L.de Galan, J.Chromatogr., 149(1978)519.

4.19 H.B.Patel and T.M.Jefferies, J.Chromatogr., 389(1987)21.

4.20 N.Tanaka, H.Goodell and B.L.Karger, J. Chromatogr., 158(1978)233.

4.21 P.J.Schoenmakers, H.A.H.Billiet, and L.de Galan, J.Chromatogr., 185(1979)179.

4.22 H.R.Neave, "Statistical Tables", George Allen and Unwin, London, 1978, 44p.

4.23 C.Hansch and A.Leo, "Substituent Constants for Correlation Analysis in Chemistry and Biology", Wiley-Interscience, New York, 1979.

5.1 R.J.Laub and J.H.Purnell, J. Chromatogr., 112(1975)71.

5.2 S.J.Custanzo, J. Chromatogr., 314(1984)402.

5.3 S.N.Deming and M.L.H.Turoff, Anal. Chem., 50(1978)546.

5.4 W.P.Price and S.N.Deming, Anal. Chim. Acta, 108(1979)227.

5.5 P.Jones and C.A.Wellington, J. Chromatogr., 213(1981)357.

5.6 C.M.Noyes, J. Chromatogr., 266(1983)451.

5.7 H.J.Issaq, G.M.Musclik and G.M.Janini, J. Liq. Chromatogr., 6(1983) 259.

5.8 A.J.Hsu, R.J.Laub and S.J.Madden, J. Liq. Chromatogr., 7(1984)615.

5.9 H.Colin, A.Krstulovic, G.Guichon and J.P.Bounine, Chromatographia, 17(1983)209.

5.10 S.Toon amd M.Rowland, J. Chromatogr., 208(1981)391.

5.11 B.Sachok, J.J.Stranahan and S.N.Deming, Anal.Chem, 53(1981)70.

5.12 B.Sachok, R.C.Kong and S.N.Deming, J. Chromatogr., 199(1980)317.

5.13 J.W.Weyland, C.H.P.Bruins and D.A.Doornbos, J. Chromatogr.s, 22(1984)31.

5.14 J.R.Gant, J.W.Dolan and L.R.Snyder, J. Chromatogr., 185(1979)153.

5.15 J.L.Glajch, J.J.Kirkland, K.M.Squire and J.M.Minor, J. Chromatogr., 199(1980)57.

5.16 P.E.Antle, Chromatographia, 15(1982)277.

5.17 J.W.Weyland, H.Rolink and D.A.Doornbod, J. Chromatogr., 247(11982)221.

5.18 H.J.Issaq, J.R.Klose, K.L.McNitt, J.E.Haky and G.M. Muschik, J. Liq. Chromatogr., 4(1981) 2091.

5.19 M.Otto and W.Wegscheider, J. Chromatogr., 258(1983)11.

5.20 M.Otto and W.Wegscheider, J. Liq. Chromatogr., 6(1983)685.

5.21 G.d'Agostino, FMitchell, L.Castagnetta and M.J.O'Hare, J. Chromatogr., 305(1984)13.

5.22 V.Svoboda, J. Chromatogr., 201(1980)241.

5.23 P.J.Schoenmakers, A.C.J.H.Drouen, H.A.H.Billiet and L.de Galan, Chromatographia, 15(1982) 688.

5.24 A.C.J.H.Druoen, P.J.Schoenmakers, H.A.H.Billiet and L.de Galan, Chromatographia, 16(1982)48.

5.25 H.A.H.Billiet, A.C.J.H.Druoen and L.de Galan, J. Chromatogr., 316(1984)231.

5.26 A.C.J.H.Druoen, H.A.H.Billiet and L.de Galan, J. Chromatogr., 352(1986)127.

5.27 P.R.Haddad, A.C.J.H.Drouen, H.A.H.Billiet and L.de Galan., J. Chromatogr., 282(1983)71.

5.28 E.P.Lankamayar and W.Wegscheider, 8th ISCLC, New York, May 21-25, 1984, paper no 3a.31.

5.29 K.Jinno and K.Kawasaki, Anal. Chim. Acta 152 (1983)25.

5.30 K.Jinno and K.Kawasaki, Chromatographia, 18,(1984)211.

5.31 K.Jinno and K.Kawasaki J.Chromatogr., 298(1984)326.

5.32 R.Kaiser, "Gas Chromatographie", Geest and Portig: Leipzig, East Germany, 1960, p33.

5.33 C.E.Goewie, J. Liq. Chromatogr., 9(1986)1431.

5.34 P.J.Schoenmakers, "Optimisation of Chromatographic Selectivity, aguide to method delvelopment", J. Chromatogr. Library, Vol.35., Elsevier Sci. Pub., Amsterdam, 1986, Chapter 4.

5.35 G. d'Agostino, L.Castagnetta, F.Mitchell and M.J.O'Hare, J. Chromatogr., 338(1985)1.

5.36 H.J.G.Bebets, B.L.Bajema and D.A.Doornbos, Anal. Chim. Acta, 151(1983) 131.

5.37 A.F.Fell, B.J.Clark and P.O.Scott, J. Chromatogr., 297(1984)203.

5.38 J.R.Gant and P.R.Perrone, Inst. Lab., 15(1985)24.

5.39 H.Scheffe, J.R.Stat.Soc., B,20(1958)344.

5.40 P.J.Schoenmakers, H.A.H.Billiet and L.de Galan, J. Chromatogr., 205(1981)13.

5.41 P.R.Haddad and S.Sekulic, J. Chromatogr., 392(1987)65.

5.42 H.B.Patel and T.M.Jefferies, J. Chromatogr., 389(1987)21.

5.43 P.J.Clarigbold, Biometrics, 11(1955)174

5.44 R.D.Snee, Chemtech, 9(1979)702

5.45 I.S.Kurotori, Industrial Quality Control, 22(1955)592

5.46 J.C.Berridge, Trends Anal. Chem., 3(1984)5.

5.47 J.C.Berridge, Chromatographia, 16(1982)172.

5.48 J.C.Berridge, J. Chromatogr., 244(1982)1.

5.49 Ref.5.34, Chapter 5,p 229.

5.50 W.H.Press, B.P.Flannery, S.A.Teklosky and W.T.Vetterling, "Numerical Recipes - the art of scientific computing", Cambridge University Press, Cambridge, 1986, 77p.

5.51 J.A.Nelder and R.Mead, Compu. J., 7(1965)308.

5.52 S.R.Bakalyar, R.McIlwrick and E.Roggendroff, J. Chromatogr., 142(1977)353.

5.53 S.Sekulic, P.R.Haddad and C.J.Lamberton, J. Chromatogr.,368(1986)125.

5.54 H.K.Smith, W.L.Switzer, G.W.Martin, S.A.Benzera, W.P.Wilson and G.W.Dixon, J.Chrom.Sci, 24(1986)70.

5.55 G.G.R.Seaton, B.J.Clark and A.F.Fell, paper presented at 11th ISCLC, July 1987, Amsterdam, Th-L-13.

5.56 B.G.M.Vandeginste, paper presented at 11th ISCLC, July 1987, Amsterdam, Th-L-11.

APPENDIX A

Programme STRENGTH

```
PROGRAM  STRENGTH (input,output);

{
{PROGRAM DESCRIPTION:

This program calculates eluotropic strength in terms of logPs
and then calculates isoeluotropic compositions of MeOH,ACN
and THF-Water mixtures. You supply the known composition of
mobile phase.
                                AUTHOR:        Hasmukh B. Patel}
LABEL
    10,20;
VAR
    amx,ax,tx,xps,vm,va,vt                          :real;
    one,two,three                                   :real;
FUNCTION alogps( am,a,t:real):real;
VAR
    w,amolm,amola,amolt,amolw,amols                 :real;
    amfac,amlp,afac,alp,tfac,tlp,wfac,wlp           :real;
BEGIN
    amfac:=  0.7928/32.04; (*MeOH*)
    amlp :=  -0.82;
    afac :=  0.7871/41.05; (*ACN*)
    alp  :=  -0.34;
    tfac :=  0.8719/72.1;  (*THF*)
    tlp  :=  +0.46;
    wfac :=  1.000/18.0;   (*H2O*)
    wlp  := -1.38;
    w    :=100-am-a-t;
    amolm:=am*amfac;
    amola:= a*afac;
    amolt:= t*tfac;
    amolw:= w*wfac;
    amols:=amolm+amola+amolt+amolw;
    alogps:=
    (amolw*wlp+amola*alp+amolm*amlp+amolt*tlp)/amols
END;
FUNCTION fps( xx,sn:real):real;
VAR
    zz:real;
```

```
BEGIN
    zz:=0.0;
    if sn=1 then fps:=alogps(xx,zz,zz);
    if sn=2 then fps:=alogps(zz,xx,zz);
    if sn=3 then fps:=alogps(zz,zz,xx);
END;
FUNCTION vol( olps,sn:real):real;
LABEL
    1,2;
VAR
    x1,x2,x3,fx1,fx2,fx3,sav,opp        :real;
BEGIN
    x1:=0;
    x2:=100;
1:  fx1:=olps-fps(x1,sn);
    sav:=fx1;
    fx2:=olps-fps(x2,sn);
    x3:=x2-fx2*(x2-x1)/(fx2-fx1);
    fx3:=olps-fps(x3,sn);
    if abs(fx3) < 1e-6 then goto 2;
    opp:=abs(fx3)/fx3+abs(sav)/sav;
    if x3>100 then    writeln('System Diverging');
    if x3<  0 then    writeln('System Diverging');
    if  fx1*fx3 < 0.0 then
    begin
    x2:=x3;
    fx2:=fx3;
    if opp > 0.0 then fx1:=fx1/2.0
    end;
    if fx1*fx3 >0.0 then
    begin
    x1:=x3;
    fx1:=fx3;
    if opp > 0.0 then fx2:=fx2/2.0
    end;
    sav:=fx3;
    goto 1;
2:  vol:=x3
END;
BEGIN
    one:=1.0;
    two:=2.0;
    three:=3.0;
10: writeln('Give MeOH%,ACN%,THF%');
    readln (amx,ax,tx);
    if amx < 0.0 then goto 20;
    xps:=alogps(amx,ax,tx);
    vm:=vol(xps,one);
    va:=vol(xps,two);
    vt:=vol(xps,three);
    writeln('logPs=',xps:5:3,'   ',
    'MeOH%=',vm:5:2,'   ',
    'ACN%=',va:5:2,'   ',
    'THF%=',vt:5:2);
    goto 10;
20: writeln('STOP');
END.
```

APPENDIX B

Programme ORIENT

```
program ORIENT (input,output,infile,outfile);
{PROGRAM DESCRIPTION:

This program ORIENT which is acronym for

        O ptimum
        R esolution
        I nvestigation using
        E xperimental  and
        N umerical
        T echniques

is designed to take data from 15 point (or 6 point for lower
resolution) experimental data for COF/CRF and corresponding
compositions to generate a factorial matrix. An interpolation
routine then uses this matrix to compute Response Factor (RF)
for the desired composition. The optimum is located by a
simplex algorithm starting from various initial compositions.
AUTHOR:     Hasmukh B. Patel                                  }
LABEL
    99;
CONST
    spc='  ';
    marg='          ';
    mi=5;
    ni=5;
TYPE
    glnarray=array[1..ni]of real;
    glmarray=array[1..mi]of real;
    glmbyn  =array[1..mi,1..ni]of real;
    glnp    =array[1..2]of real;
    starray =array[1..2]of real;
VAR
    infile              :text;
    outfile             :text;
    indat,outdat        :varying [25] of char;
    x1a                         :glnarray;
    x2a                         :glmarray;
    ya                          :glmbyn;
    A                           :array[1..8,1..15]of real;
```

```
    opt                          :array[1..8]of real;
    step,start,xmin                              :starray;
    x1star,x2star,reqmin,ynewlo,optim,bpc,cpc    :real;
    preopt,delta,dtol,stepup                        :real;
    l,k,j,i,n,konvge,kcount,numres,ifault,icount:integer;
PROCEDURE POLINT
    (xa,yy:glnarray;n:integer;
    x                :real;
    var     y,dy             :real);
VAR
    ns,m,i               :integer;
    w,hp,ho,dift,dif,den    :real;
    c                           :glmarray;
    d                           :glnarray;
BEGIN
    ns:=1;
    dif:=abs(x-xa[1]);
    for i:=1 to n do
    begin
    dift:=abs(x-xa[i]);
    if (dift < dif) then
    begin
        ns:=i;
        dif:=dift;
    end;
    c[i]:=yy[i];
    d[i]:=yy[i];
    end;
    y:=yy[ns];
    ns:=ns-1;
    for m:=1 to n-1 do
    begin
    for i:=1 to n-m do
    begin
        ho:=xa[i]-x;
        hp:=xa[i+m]-x;
        w:=c[i+1]-d[i];
        den:=ho-hp;
        if (den=0.0) then
        begin
            writeln('Pause in routine POLINT');
            readln;
        end;
        den:=w/den;
        d[i]:=hp*den;
        c[i]:=ho*den;
    end;
    if ((2*ns) < (n-m)) then
    begin
        dy:=c[ns+1]
    end
    else
    begin
        dy:=d[ns];
        ns:=ns-1
    end;
    y:=y+dy;
    end
```

```
END;
PROCEDURE POLIN2
    (m,n    :integer;
    x1,x2   :real;
var y,dy    :real);
VAR
    k,j             :integer;
    ymtmp   :glmarray;
    yntmp   :glnarray;
BEGIN
    for j:=1 to m do
    begin
    for k:=1 to n do
    begin
        yntmp[k]:=ya[j,k]
    end;
    polint(x2a,yntmp,n,x2,ymtmp[j],dy)
    end;
    polint(x1a,ymtmp,m,x1,y,dy)
END;
FUNCTION FN(x1x2:glnp):real;
LABEL
    99;
VAR
    y,dy,x1,x2    :real;
    mi,ni     :integer;
BEGIN
    x1:=x1x2[1];
    x2:=x1x2[2];
    mi:=5;
    ni:=5;
    if (x1 > x1a[5]) or (x1 < x1a[1]) then
    begin
    fn:=1e32;
    goto 99;
    end;
    if (x2 > x2a[5]) or (x2 < x2a[1]) then
    begin
    fn:=1e32;
    goto 99;
    end;
    if ((x1+x2) > 2*x1a[5]) then
    begin
    fn:=1e32;
    goto 99;
    end;
    polin2 (mi,ni,x1,x2,y,dy);
    delta:=abs(preopt-(-y));
    if (delta > dtol) then
    begin
    step[1]:=step[1]*2;
    step[2]:=step[2]*2;
    end;
    if (delta <= dtol) then
    begin
    step[1]:=1.0;
    step[2]:=1.0;
    end;
```

```
    if step[1] > stepup then
    begin
    step[1]:=stepup;
    step[2]:=stepup;
    end;
    fn:=-y;
    preopt:=(-y);
99:END;
PROCEDURE NELMIN
    (n, konvge, kcount    : integer;
    var icount, numres    : integer;
    ifault                : integer;
    start,step              : starray;
    var xmin              : starray;
    var ynewlo            : real;
        reqmin              : real);
LABEL
    10,20,30,40,43,47,50,70,80,90,100,110,
    130,133,137,140,150,160,165,170,180,182,
    185,188,190,200,230,235,240,250,260,270,
    280,290,300,310;
VAR
    p         :array[1..2,1..3]of real;
    pstar :array[1..2]of real;
    p2star:array[1..2]of real;
    pbar  :array[1..2]of real;
    y     :array[1..3]of real;
    dn,z,ylo,rcoeff,ystar,ecoeff,y2star,ccoeff      :real;
    rq,x,del,one,half,zero,eps,dnn          :real;
    jcount,ilo,ihi,l,nn,j,i,k                   :integer;
BEGIN
    rcoeff:=1.0;
    ecoeff:=2.0;
    ccoeff:=0.5;
    one   :=1.0;
    half  :=0.5;
    zero  :=0.0;
    eps   :=0.001;
{  validity check   }
    ifault:=1;
    ifault:=2;
    icount:=0;
    numres:=0;
    jcount:=konvge;
    dn:=n;
    nn:=n+1;
    dnn:=nn;
    del:=one;
    rq:=reqmin*dn;
{   init simplex    }
10: for i:=1 to n do
    begin
    p[i,nn]:=start[i];
20: end;
    y[nn]:=fn(start);
    for j:=1 to n do
    begin
    x:=start[j];
```

```
    start[j]:=start[j]+step[j]*del;
    for i:=1 to n do
    begin
        p[i,j]:=start[i];
30: end;
    y[j]:=fn(start);
    start[j]:=x;
40: end;
    icount:=icount + nn;
{   simplex construct       }
43: ylo:=y[1];
    ilo:=1;
    for i:=2 to nn do
    begin
    if y[i] >= ylo then goto 47;
    ylo:=y[i];
    ilo:=i;
47: end;
50: ynewlo:=y[1];
    ihi:=1;
    for i:=2 to nn do
    begin
    if y[i] <= ynewlo then goto 70;
    ynewlo:=y[i];
    ihi:=i;
70: end;

{   centroid of simplex     }
    for i:=1 to n do
    begin
    Z:=zero;
    for j:=1 to nn do
    begin
        z:=z + p[i,j];
80: end;
    z:=z - p[i,ihi];
    pbar[i]:=z/dn;
90: end;
{   reflection thro' centroid       }
    for i:=1 to n do
    begin
    pstar[i]:=pbar[i] + rcoeff*(pbar[i]-p[i,ihi]);
100:end;
    ystar:=fn(pstar);
    icount:=icount+1;
    if ystar >= ylo then goto 140;
{   extension       }
    for i:=1 to n do
    begin
    p2star[i]:=pbar[i]+ecoeff*(pstar[i]-pbar[i]);
110:end;
    y2star:=fn(p2star);
    icount:=icount+1;
{   check extension }
    if y2star >= ystar then goto 133;
{   retain reflection       }
    for i:=1 to n do
    begin
```

```
    p[i,ihi]:=p2star[i];
130:end;
    y[ihi]:=ystar;
    goto 230;
{   reatain reflection      }
133:for i:=1 to n do
    begin
    p[i,ihi]:=pstar[i];
137:end;
    y[ihi]:=ystar;
    goto 230;
{   no extension    }
140:l:=0;
    for i:=1 to nn do
    begin
    if y[i] > ystar then l:=l+1;
150:end;
    if l > 1 then goto 133;
    if l = 0 then goto 170;
{   contraction on reflection side of centroid      }
    for i:=1 to n do
    begin
    p2star[i]:=pbar[i] +ccoeff*(pstar[i]-pbar[i]);
160:end;
    y2star:=fn(p2star);
    icount:=icount+1;
    if y2star <= ystar then goto 182;
{   retain reflection       }
    for i:=1 to n do
    begin
    p[i,ihi]:=pstar[i];
165:end;
    y[ihi]:=ystar;
    goto 230;
{   contn. on y[ihi] side of centroid       }
170:for i:=1 to n do
    begin
    p2star[i]:=pbar[i]+ccoeff*(p[i,ihi]-pbar[i]);
180:end;
    y2star:=fn(p2star);
    icount:=icount+1;
    if y2star > y[ihi] then goto 188;
{   retain contrection      }
182:for i:=1 to n do
    begin
    p[i,ihi]:=p2star[i];
185:end;
    y[ihi]:=y2star;
    goto 230;
{   contract whole simplex  }
188:for j:=1 to nn do
    begin
    for i:=1 to n do
    begin
        p[i,j]:=(p[i,j]+p[i,ilo])*half;
        xmin[i]:=p[i,j];
190:        end;
    y[j]:=fn(xmin);
```

```
200:end;
    icount:=icount+nn;
    if icount > kcount then goto 260;
    goto 43;
{   check if ylo improved   }
230:if y[ihi] >= ylo then goto 235;
    ylo:=y[ihi];
    ilo:=ihi;
235:jcount:=jcount-1;
    if jcount <> 0 then goto 50;
{   check if minimum reached        }
    jcount:=konvge;
    z:=zero;
    for i:=1 to nn do
    begin
    z:=z+y[i];
240:end;
    x:=z/dnn;
    z:=zero;
    for i:=1 to nn do
    begin
    z:=z+(y[i]-x)*2;
250:end;
    if z > rq then goto 50;
{   check loc/glob minim.   }
260:for i:=1 to n do
    begin
    xmin[i]:=p[i,ilo];
270:end;
    ynewlo:=y[ilo];
    if icount > kcount then goto 310;
    for i:=1 to n do
    begin
    del:=step[i]*eps;
    xmin[i]:=xmin[i]+del;
        z:=fn(xmin);
    icount:=icount+1;
    if z < ynewlo then goto 290;
    xmin[i]:=xmin[i]-del-del;
    z:=fn(xmin);
        icount:=icount+1;
    if z < ynewlo then goto 290;
    xmin[i]:=xmin[i]+del;
280:end;
    ifault:=0;
    goto 310;
{   restart procedure       }
290:for i:=1 to n do
    begin
    start[i]:=xmin[i];
300:end;
    del:=eps;
    numres:=numres+1;
    goto 10;
310:END;
{    MAIN program   }
BEGIN
    writeln('Name of data file ?');
```

```
readln (indat);
open   (infile,indat,old);
reset  (infile);
writeln('Name of output file ?');
readln (outdat);
open   (outfile,outdat,new);
rewrite(outfile);
readln (infile,x1a[1],x1a[2],x1a[3],x1a[4],x1a[5]);
x2a[1]:=x1a[1];
x2a[2]:=x1a[2];
x2a[3]:=x1a[3];
x2a[4]:=x1a[4];
x2a[5]:=x1a[5];
for j:=1 to 2 do
begin
    writeln;
writeln(outfile);
end;
writeln(        marg,
    'DATA FILE ',outdat,' CREATED FROM ',indat);
writeln(outfile,marg,
    'DATA FILE ',outdat,' CREATED FROM ',indat);
writeln;
writeln(outfile);
writeln(outfile,
marg,'B% I     >--------------C%------------------->');
writeln(
marg,'B% I     >--------------C%------------------->');
writeln(outfile,
marg,'   I                                          ');
writeln(
marg,'   I                                          ');
writeln(outfile,marg,' V  ':6,spc,
    x1a[1]:6:2,spc,
    x1a[2]:6:2,spc,
    x1a[3]:6:2,spc,
    x1a[4]:6:2,spc,
    x1a[5]:6:2);
writeln(        marg,'  V ':6,spc,
    x1a[1]:6:2,spc,
    x1a[2]:6:2,spc,
    x1a[3]:6:2,spc,
    x1a[4]:6:2,spc,
    x1a[5]:6:2);
writeln(outfile);
writeln;
for j:=1 to mi do
begin
readln(infile,
    ya[j,1],
    ya[j,2],
    ya[j,3],
    ya[j,4],
    ya[j,5]);
writeln(outfile,marg,
    x1a[j]:6:2,spc,
    ya[j,1]:6:2,spc,
    ya[j,2]:6:2,spc,
```

```
    ya[j,3]:6:2,spc,
    ya[j,4]:6:2,spc,
    ya[j,5]:6:2);
writeln(        marg,
    xla[j]:6:2,spc,
    ya[j,1]:6:2,spc,
    ya[j,2]:6:2,spc,
    ya[j,3]:6:2,spc,
    ya[j,4]:6:2,spc,
    ya[j,5]:6:2);
end;
writeln;
writeln(outfile);
n:=2; numres:=0;        icount:=0;  ifault:=0;
konvge:=5;
reqmin:=1e-16;
writeln(            marg,'Maximum iterations ?');
readln (kcount);
stepup:=100;
dtol:=0.00001;
writeln(            marg,
    'Maximum iterations        =',kcount:6);
writeln(outfile,marg,
    'Maximum iterations        =',kcount:6);
writeln(        marg,
    'Tolerance for step change =',dtol:12:8);
writeln(outfile,marg,
    'Tolerance for step change =',dtol:12:8);
writeln(        marg,
    'Maximum step size         =',stepup:6:0);
writeln(outfile,marg,
    'Maximum step size         =',stepup:6:0);
writeln(outfile);
writeln;
writeln
(outfile,marg,
    'Bi%':6,spc,
    'Ci%':6,spc,
    'COF(Cal)':12,spc,
    'B%':6,spc,
    'C%':6,spc,
    'A%':6,spc,
    'Rest.':5,spc,
    'Itrs.':5);
writeln(outfile);
writeln
(       marg,
    'Bi%':6,spc,
    'Ci%':6,spc,
    'COF(Cal)':12,spc,
    'B%':6,spc,
    'C%':6,spc,
    'A%':6,spc,
    'Rest.':5,spc,
    'Itrs.':5);
writeln;
step[1]:=1.0;
step[2]:=1.0;
```

```
j:=0;
while not eof(infile) do
begin
readln(infile,start[1],start[2]);
j:=j+1;
x1star:=start[1];
x2star:=start[2];
preopt:=0;
NELMIN
(n, konvge, kcount, icount, numres, ifault,
start,step,xmin,ynewlo,reqmin);
bpc    := xmin[1];
cpc    := xmin[2];
optim :=-ynewlo;
writeln(
        marg,x1star:6:2,spc,
        x2star:6:2,spc,
        optim:12:8,spc,
        bpc:6:2,spc,
        cpc:6:2,spc,
        100-bpc-cpc:6:2,spc,
        numres:5,spc,
        icount:5);
writeln(outfile,
        marg,x1star:6:2,spc,
        x2star:6:2,spc,
        optim:12:8,spc,
        bpc:6:2,spc,
        cpc:6:2,spc,
        100-bpc-cpc:6:2,spc,
        numres:5,spc,
        icount:5);
a[1,j]:=x1star;
a[2,j]:=x2star;
a[3,j]:=optim;
a[4,j]:=bpc;
a[5,j]:=cpc;
a[6,j]:=100-bpc-cpc;
a[7,j]:=numres;
a[8,j]:=icount;
end;
writeln;
writeln(outfile);
writeln(        marg,
'Sorted COF values and corresponding data');
writeln(outfile,marg,
'Sorted COF values and corresponding data');
writeln;
writeln(outfile);
for k:=2 to 15 do
begin
for l:=1 to 8 do
begin
    opt[l]:=a[l,k]
end;
for i:=k-1 downto 1 do
begin
        if (a[3,i] >= opt[3] ) then goto 99;
```

```
                for l:=1 to 8 do
                begin
                    a[l,i+1]:=a[l,i]
                end;
        end;
    i:=0;
99: ;
    for l:=1 to 8 do
    begin
       a[l,i+1]:=opt[l]
    end;
    end;
    for j:=1 to 15 do
    begin
    writeln(
    marg,   a[1,j]:6:2,spc,
            a[2,j]:6:2,spc,
            a[3,j]:12:8,spc,
            a[4,j]:6:2,spc,
            a[5,j]:6:2,spc,
            a[6,j]:6:2,spc,
            a[7,j]:5:0,spc,
            a[8,j]:5:0);
    writeln(outfile,
    marg,   a[1,j]:6:2,spc,
            a[2,j]:6:2,spc,
            a[3,j]:12:8,spc,
            a[4,j]:6:2,spc,
            a[5,j]:6:2,spc,
            a[6,j]:6:2,spc,
            a[7,j]:5:0,spc,
            a[8,j]:5:0);
    end;
    close(outfile);
    close(infile)
END.
```

APPENDIX C

Programme NUMEROGRAPH

```
program NUMEROGRAM (input,output,infile,outfile);
const
    mi=5;
    ni=5;
    mats=25;
type
    glnarray=array[1..ni]of real;
    glmarray=array[1..mi]of real;
    glmbyn  =array[1..mi,1..ni]of real;
    glnp    =array[1..2]of real;
    starray =array[1..2]of real;
var
    infile,outfile      :text;
    indat,outdat        :varying [25] of char;
    x1a                         :glnarray;
    x2a                         :glmarray;
    ya                          :glmbyn;
    start               :starray;
    rmat                :array[0..mats,0..mats]of real;
    Rs, Rd              :real;
    j,i,n,pair,pairs            :integer;
    max,min,trmat,scf           :real;
procedure POLINT
    (xa,yy:glnarray;n       :integer;
    x               :real;
    var     y,dy            :real);
var
    ns,m,i              :integer;
    w,hp,ho,dift,dif,den    :real;
    c                           :glmarray;
    d                           :glnarray;
begin
    ns:=1;
    dif:=abs(x-xa[1]);
    for i:=1 to n do
    begin
    dift:=abs(x-xa[i]);
    if (dift < dif) then
    begin
        ns:=i;
```

```
        dif:=dift;
    end;
    c[i]:=yy[i];
    d[i]:=yy[i];
    end;
    y:=yy[ns];
    ns:=ns-1;
    for m:=1 to n-1 do
    begin
    for i:=1 to n-m do
    begin
        ho:=xa[i]-x;
        hp:=xa[i+m]-x;
        w:=c[i+1]-d[i];
        den:=ho-hp;
        if (den=0.0) then
        begin
            writeln('Pause in routine POLINT');
            readln;
        end;
        den:=w/den;
        d[i]:=hp*den;
        c[i]:=ho*den;
    end;
    if ((2*ns) < (n-m)) then
    begin
        dy:=c[ns+1]
    end
    else
    begin
        dy:=d[ns];
        ns:=ns-1
    end;
    y:=y+dy;
    end
end;
procedure POLIN2
    (x1a    :glnarray;
    x2a             :glmarray;
    ya              :glmbyn;
    m,n             :integer;
    x1,x2   :real;
var y,dy    :real);
var
    k,j             :integer;
    ymtmp   :glmarray;
    yntmp   :glnarray;
begin
    for j:=1 to m do
    begin
    for k:=1 to n do
    begin
        yntmp[k]:=ya[j,k]
    end;
    polint(x2a,yntmp,n,x2,ymtmp[j],dy)
    end;
    polint(x1a,ymtmp,m,x1,y,dy)
end;
```

```
function FN(x1x2:glnp):real;
var
    y,dy    :real;
begin
    polin2 (x1a,x2a,ya,mi,ni,x1x2[1],x1x2[2],y,dy);
    fn:=y;
end;
{    MAIN program   }
BEGIN
    write('How many pairs of peaks ? ');
    readln (pairs);
    write('Desired resolution Rd ? ');
    readln(Rd);
        write('Name of output file ? ');
        readln (outdat);
        open   (outfile,outdat,new);
        rewrite(outfile);
    for i:=0 to mats do
    begin
        for j:=0 to mats do
        begin
            rmat[i,j]:= pairs;
        end;
    end;
    for pair:=1 to pairs do
    begin
        write('Name of data file (.dat assumed) ? ');
        readln (indat);
    indat:=indat + '.dat';
        open   (infile,indat,old);
        reset  (infile);
        readln(infile,x1a[1],x1a[2],x1a[3],x1a[4],x1a[5]);
        x2a[1]:=x1a[1];
        x2a[2]:=x1a[2];
        x2a[3]:=x1a[3];
        x2a[4]:=x1a[4];
        x2a[5]:=x1a[5];
        for j:=1 to mi do
        begin
            readln(infile,
                ya[j,1],ya[j,2],ya[j,3],ya[j,4],ya[j,5]);
    end;
    close(infile);
    n:=2;
    max:=-1e32;
    min:=+1e32;
    for i:=0 to mats do
    begin
        for j:=0 to mats do
        begin
            start[1]:=j*100/mats;
            start[2]:=i*100/mats;
            trmat:= fn(start);
            if trmat > max then max:=trmat;
            if trmat < min then min:=trmat;
        end;
    end;
    scf:=max-min;
```

```
    for i:=0 to mats do
    begin
        for j:=0 to mats do
        begin
            start[1]:=j*100/mats;
            start[2]:=i*100/mats;
            Rs:= fn(start);
            trmat:= (Rs-min)/scf;
            if Rs < Rd then
            begin
                if trmat < 0.8 then
                    rmat[i,j]:=rmat[i,j]-1.0
            end;
        end;
    end;
    for i:=0 to mats do
    begin
    write(outfile,i*4:4,'     ');
    write(         i*4:4,'     ');
    for j:=0 to mats do
    begin
        write(outfile,rmat[i,j]:1:0);
        write(         rmat[i,j]:1:0)
    end;
    writeln;
    writeln(outfile);
    end;
    for i:=1 to 5 do
    begin
    writeln;
    writeln(outfile)
    end;
    end;
    close(outfile);
end.
```

APPENDIX D

Programme SIMULA

```
program  SIMULA  (input,output,infile,outfile);
label
    88,99;
const
    spc='   ';
    marg='                ';
    mi=5;
    ni=5;
type
    glnarray=array[1..ni]of real;
    glmarray=array[1..mi]of real;
    glmbyn  =array[1..mi,1..ni]of real;
    glnp    =array[1..2]of real;
    starray =array[1..2]of real;
var
    infile              :text;
    outfile             :text;
    indat,outdat,rf     :varying [25] of char;
    x1a                         :glnarray;
    x2a                         :glmarray;
    ya                          :glmbyn;
    pk                          :array[1..20,1..mi,1..ni]of real;
    start               :starray;
    optim                       :real;
    j,n,k,l,i,peak,pks          :integer;
procedure POLINT
    (xa,yy:glnarray;n       :integer;
    x               :real;
    var     y,dy            :real);
var
    ns,m,i              :integer;
    w,hp,ho,dift,dif,den    :real;
    c                           :glmarray;
    d                           :glnarray;
begin
    ns:=1;
    dif:=abs(x-xa[1]);
    for i:=1 to n do
    begin
    dift:=abs(x-xa[i]);
```

```
    if (dift < dif) then
    begin
        ns:=i;
        dif:=dift;
    end;
    c[i]:=yy[i];
    d[i]:=yy[i];
    end;
    y:=yy[ns];
    ns:=ns-1;
    for m:=1 to n-1 do
    begin
    for i:=1 to n-m do
    begin
        ho:=xa[i]-x;
        hp:=xa[i+m]-x;
        w:=c[i+1]-d[i];
        den:=ho-hp;
        if (den=0.0) then
        begin
            writeln('Pause in routine POLINT');
            readln;
        end;
        den:=w/den;
        d[i]:=hp*den;
        c[i]:=ho*den;
    end;
    if ((2*ns) < (n-m)) then
    begin
        dy:=c[ns+1]
    end
    else
    begin
        dy:=d[ns];
        ns:=ns-1
    end;
    y:=y+dy;
    end
end;
procedure POLIN2
(   m,n             :integer;
    x1,x2   :real;
var y,dy    :real);
var
    k,j             :integer;
    ymtmp   :glmarray;
    yntmp   :glnarray;
begin
    for j:=1 to m do
    begin
    for k:=1 to n do
    begin
        yntmp[k]:=ya[j,k]
    end;
    polint(x2a,yntmp,n,x2,ymtmp[j],dy)
    end;
    polint(x1a,ymtmp,m,x1,y,dy)
end;
```

```
function FN(x1x2:glnp):real;
var
    y,dy,x1,x2    :real;
begin
    x1:=x1x2[1];
    x2:=x1x2[2];
    polin2 (mi,ni,x1,x2,y,dy);
    fn:=y;
end;
{    MAIN program   }
BEGIN
    write('What is RF k/COF/ORM ? ');
    readln(rf);
    writeln('Name of output file ?');
    readln (outdat);
    open   (outfile,outdat,new);
    rewrite(outfile);
    write('How many peaks ? ');
    readln(pks);
    for l:=1 to pks do
    begin
    write('Data file (ext .dat assumed) ',l,' ? ');
    readln(indat);
    indat:=indat+'.dat';
    open   (infile,indat,old);
    reset  (infile);
    readln(infile,x1a[1],x1a[2],x1a[3],x1a[4],x1a[5]);
    x2a[1]:=x1a[1];
    x2a[2]:=x1a[2];
    x2a[3]:=x1a[3];
    x2a[4]:=x1a[4];
    x2a[5]:=x1a[5];
    writeln(        'Data file ',indat,' read.');
    writeln(outfile,'Data file ',indat,' read.');
    for j:=1 to mi do
    begin
    for k:=1 to ni do
    begin
        read(infile,pk[l,j,k]);
    end;
    readln(infile);
    end;
    close(infile);
    end;
    n:=2;
    writeln(        marg,
              'Peak ':6,spc, 'B%':6,spc, 'C%':6,spc, rf:14);
    writeln(outfile,marg,
              'Peak ':6,spc, 'B%':6,spc, 'C%':6,spc, rf:14);
88: write ('B% = ');
    readln(start[1]);
    if start[1] < 0 then goto 99;
    write ('C% = ');
    readln(start[2]);
    for peak:=1 to pks do
    begin
        for i:=1 to mi do
    begin
```

```
        for j:=1 to ni do
        begin
            ya[i,j]:=pk[peak,i,j]
        end;
    end;
    optim:=fn(start);
    writeln(        marg,
              peak:6,spc, start[1]:6:2,spc,start[2]:6:2,spc,
              optim:14:8);
    writeln(outfile,marg,
              peak:6,spc, start[1]:6:2,spc,start[2]:6:2,spc,
              optim:14:8)
    end;
    goto 88;
99: close(outfile);
end.
```